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Ecological Effects and Genetic Diversity of the Invading Rhizocephalan Parasite Loxothylacus panopaei in the Flatback Mud Crab Eurypanopeus depressus

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ECOLOGICAL EFFECTS AND GENETIC DIVERSITY OF THE INVADING RHIZOCEPHALAN PARASITE *LOXOTHYLACUS PANOPAEI* IN THE FLATBACK MUD CRAB *EURYPANOPEUS DEPRESSUS*

by

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ABSTRACT

The rhizocephalan barnacle *Loxothylacus panopaei* (Gissler, 1884) is a parasite of xanthid crabs. Infection results in parasitic castration and anecdysis of the host. *Loxothylacus panopaei* is invasive to the US Atlantic coast. The parasite's range was expanded by introduction of infected crabs to Chesapeake Bay in the mid-1960s, and now extends from Long Island Sound, New York to Cape Canaveral, Florida.

Monthly flatback mud crab (*Eurypanopeus depressus* Smith, 1869) collections over 13 months (January 2012–January 2013) at three South Carolina locations found an overall parasite prevalence of 24.2% (391 of 1,615), and provided the first reports of *L. panopaei* at Waties Island, Murrells Inlet and North Inlet. North and South Carolina parasite DNA sequence analysis revealed the presence of four mitochondrial DNA cytochrome *c* oxidase subunit I (COI) haplotypes (one of which was a new haplotype), and indicates that the Carolina populations are the result of a range expansion from the original Atlantic coast introduction in Chesapeake Bay. To investigate ecological relationships between *Eurypanopeus depressus* hosts and their parasites, prey consumption was compared between parasitized (externa-bearing) and unparasitized (externa-lacking) *E. depressus* 8–13 mm carapace width. Parasitized crabs consumed significantly fewer (median = 2) mussels than unparasitized crabs (median = 4) over 72 hours (Kruskal-Wallis, $H = 5.94$, $d.f. = 1$, $p = 0.02$). Relationships between host size, parasite externa color, and developmental stages of the externa were examined. Of the total externae collected (n = 476), 35.5% were placed into the oldest (purple) developmental category. Size of *L. panopaei* externae tended to increase with increasing *E. depressus* carapace width (Spearman rank order correlation, $p < 0.001$, $r = 0.65$), suggesting that the externa exists based on the balance of two laws: (1) The parasitic externa must grow large enough to produce nauplii in amounts that will ensure infection of new hosts; and (2) the externa must remain small enough to allow host mobility to forage in between the small oyster crevices and avoid predation.

PREFACE

This thesis is divided into three chapters. The first chapter, "Prevalence of the invasive rhizocephalan parasite *Loxothylacus panopaei* in *Eurypanopeus depressus* and genetic relationships of the parasite in North and South Carolina" discusses *L. panopaei* origin of introduction and distribution along the US Atlantic coast by identifying specific haplotypes from North and South Carolina parasite populations. These results fill a geographic gap in *L. panopaei* mitochondrial cytochrome *c* oxidase subunit I (COI) data that extends from Long Island Sound, New York south around Florida to Chauvin, Louisiana.

The second chapter, "Reduced ecological functioning of the flatback mud crab *Eurypanopeus depressus* by infection with the invasive parasitic rhizocephalan barnacle *Loxothylacus panopaei*" reports on experiments comparing foraging rates between parasitized and unparasitized *E. depressus* using *Geukensia demissa* and *Brachidontes exustus* as the prey items.

The final chapter, "Relationships among host crab carapace width and size and coloration of *Loxothylacus panopaei* external reproductive structures" contributes a current report on North and South Carolina *L. panopaei* externae size and color, and relationship to host crab carapace width. This study follows from similar research by Wardle and Tirpak (1991) on external reproductive structures of *Loxothylacus texanus.*

INTRODUCTION

Global connectivity, often associated with the movement of commerce and people, has facilitated an increase in the number of nonindigenous species, especially since the 1950s (Carlton, 1989; Cohen and Carlton, 1997; Hulme, 2009). Translocation of aquatic and terrestrial species often decreases native biodiversity, and may cause species extinctions (Baskin, 1996; Williamson, 1996; Wilcove et al., 1998; Snyder and Evans, 2006; Hulme, 2009). Because these species often do not have natural predators and are less affected by parasites in the new environment (Torchin et al., 2001), invasives typically outcompete and displace similar native species, commonly attaining higher densities than native analogs (Snyder and Evans, 2006). Nonindigenous species also harbor diseases and parasites that are detrimental to the persistence of native species and natural assemblages (Bower et al., 1994; Williamson, 1996; Cohen et al., 1995).

Crustaceans are easily transported globally because they foul ship hulls and their pelagic larvae are transported in ship ballast water (Williamson, 1996; Cohen and Carlton, 1997; Kerckhof et al., 2010). A number of crustacean invaders have successfully established invasive populations along US coasts (Cohen and Carlton, 1997). The European green crab *Carcinus maenas* (Linnaeus, 1758) is native to the eastern Atlantic Ocean, but has been documented in the northwest Atlantic Ocean since 1989 (Cohen et al., 1995). *Petrolisthes armatus* (Gibbes, 1850), the green porcelain crab, is native to Brazil but was discovered on oyster reefs in Cape Canaveral, Florida in 1994 and has since spread north to North Inlet, South Carolina (Knott et al., 1999; Hollebone and Hay, 2007). The titan barnacle *Megabalanus coccopoma* (Darwin, 1854), native to the tropical eastern Pacific was recorded from the northern Gulf of Mexico, Georgia and the

Carolinas in the early 2000s (Yamaguchi, et al., 2009). Some of these crustacean invaders are able to cause trophic-level changes within their new habitat, as seen in the *C. maenas–Littorina obtusata* (Linnaeus, 1758) relationship. In the western Atlantic Ocean, *C. maenas* consumes *Littorina* snails in large quantities, resulting in a trophic cascade that alters the abundance of a macroalgae species (Snyder and Evans, 2006; Hulme, 2009).

Loxothylacus panopaei (Gissler, 1884) is a rhizocephalan barnacle that parasitizes xanthid mud crabs and is invasive to the US Atlantic coast (Van Engel et al., 1966; Walker, 2001). The nonindigenous range extends from Long Island Sound, New York, to just north of Cape Canaveral, Florida, while the native range occurs from Cape Canaveral south around Florida into the Gulf of Mexico and into Caribbean waters, as far east as Venezuela (Hines et al., 1997; Kruse et al., 2012; Freeman et al., 2013). In response to an increased occurrence of oyster diseases such as Dermo and MSX in Chesapeake Bay during the mid-1960s, Gulf of Mexico oysters were transported to the US Atlantic coast (Van Engel et al., 1966). Mud crabs carrying *L. panopaei* were transported with the oysters from their native range to the Chesapeake area, where they established viable populations (Hines et al., 1997; Tolley et al., 2006; Van Engel et al., 1966).

The Rhizocephala is composed of barnacles that control their hosts at the morphological, physiological and behavioral levels (Glenner et al., 2000; Walker, 2001). Parasitic anecdysis of the host results from infection (O'Brien and Van Wyk, 1985), while the endocrine and central nervous systems sustain damage from the parasitic internal rootlet system (Høeg, 1995). *Loxothylacus panopaei* infection causes parasitic castration, which transforms the abdominal morphology of males into the broad abdomen

of females, and inhibits females from producing eggs (Høeg, 1995). Infection has also been shown to significantly reduce host feeding rates (O'Shaughnessy et al., in review).

Rhizocephalan barnacles are effective parasites because they exhibit an extremely modified life cycle compared to free-living barnacles. The naupliar and cyprid larval stages are the Rhizocephala's only morphological links to the Cirripedia (Høeg, 1995), while the adult form is unique and consists of an external reproductive sac (externa) and an internal rootlet system (interna) used for nutrient up-take (Høeg, 1995). The parasite's internal rootlet system ramifies throughout the hemolymph of the host body (O'Brien and Van Wyk, 1985), absorbs nutrients and emerges from the host abdomen at maturity, forming an external reproductive sac that lacks the segmentation and calcareous plates characteristic of free-living barnacles (Walker, 2001; Glenner and Hebsgaard, 2006). At this stage it is a female virgin externa, awaiting the arrival of free-living male cyprid larvae, which are attracted by pheromones emitted by the female externa (Walker, 2001). The male cyprid enters one of the two receptacles on the female externa, fertilization occurs, and after development naupliar larvae are released from the mature externa into the water column. Free-swimming, lecithotrophic naupliar larvae develop into cyprid larvae within two days (Glenner, 2001; Walker, 2001).

Loxothylacus panopaei is dioecious: The male cyprid settles on a virgin externa for fertilization purposes, while the female cyprid settles in the branchial chamber of a potential host within 24 hour post molt (Walker et al., 1992). Upon settlement, the female cyprid metamorphoses into the kentrogon stage, and the penetration instrument—a hollow cuticular stylet—develops in a sheath within the kentrogon body. Penetration of the host occurs when the kentrogon stylet is everted into the gill lamellae, piercing the

hemocoel of the crab (Walker, 2001). The time from settlement on the host to inoculation via stylet takes 48–72 hours, and from infection to externa emergence (internal phase) is 25–42 days (Walker et al., 1992; Glenner, 2001). The mature externa releases a larval brood 14–16 days after fertilization and a brood is released every 5–6 days when water temperatures are around 25°C (Walker et al., 1992).

The first report of *L. panopaei* from the US Atlantic coast was by Van Engel et al. (1966) who observed 54% prevalence in 595 *Eurypanopeus depressus* (Smith, 1869) mud crabs at Gloucester Point, Virginia in the York River (Chesapeake Bay), and since reported from Long Island Sound in New York (11.9% prevalence, Freeman et al., 2013), Bogue Sound in North Carolina (47.4% prevalence, Hines et al., 1997), coastal Georgia (67.0% prevalence, Kruse and Hare, 2007), and the northern Florida coast (93% prevalence, Kruse et al., 2012). Within the current invasive range, only the xanthid crabs *Eurypanopeus depressus*, *Rhithropanopeus harrisii* (Gould, 1841) and *Dispanopeus sayi* (Smith, 1869) have been found with *L. panopaei* infection, while another co-existing xanthid crab on temperate intertidal oyster reefs, *Panopeus herbstii* H. Milne Edwards, 1834, remains uninfected (McDonald, 1982; Hines et al., 1997; Kruse and Hare, 2007; Kruse et al., 2012). Previous studies generally found higher *L. panopaei* prevalence in the nonindigenous range relative to the native range (Hines et at., 1997; Kruse and Hare, 2007; Kruse et al., 2012).

Prevalence variation can be attributed to a number of factors, including presence of multiple parasite lineages with different haplotypes and host specificities (Kruse and Hare, 2007; Kruse et al., 2012). Molecular markers are a useful invasion biology tool because they can identify the source population of introduction, document the expanding

invasive range, and investigate host specificity and haplotype frequency (Kruse and Hare, 2007; Kruse et al., 2012). Kruse et al. (2012) used DNA sequence analyses of the mitochondria-encoded cytochrome *c* oxidase subunit I locus (COI) to show that two distinct *L. panopaei* clades were present from Chesapeake Bay to Florida and into the Gulf of Mexico: The 'ER clade' infecting only *E. depressus* and *R. harrisii*, which consisted of hosts from both native and invasive ranges, and the 'P clade' infecting mostly *Panopeus* crabs, which was composed of parasites found exclusively on crabs in the native range. Kruse et al. (2012) concluded that the source of infection in Chesapeake Bay was from Gulf of Mexico parasites in the 'ER clade.' Although these studies provided resolution to the questions of host specificity and expansion of the nonindigenous range along most of the US Atlantic and Gulf of Mexico coasts, North and South Carolina parasite populations were not described.

Loxothylacus panopaei negatively affects its mud crab hosts at the individual and population levels (Van Engel et al., 1966; Walker, 2001). Although infection does not kill the individual host (Isaeva et al., 2001; Hines et al., 1997), previous studies have found that rhizocephalan infection indirectly reduces host fitness. Wardle and Tirpak (1991) observed that *Callinectes sapidus* Rathbun, 1896 forage less aggressively than uninfected crabs, and O'Shaughnessy et al. (in review) found that *E. depressus* infected with *L. panopaei* consumed mussels at a significantly slower rate than uninfected individuals. The sand crab *Portunus pelagicus* (Linnaeus, 1758) infected with *Sacculina granifera* Boschma, 1973 has been shown to burrow below sediment at a significantly slower rate than uninfected male *P. pelagicus* (Bishop and Cannon, 1979). Similar behavior was observed in *C. sapidus* infected with *Loxothylacus texanus* Boschma, 1933 (Wardle and

Tirpak, 1991). Changes in burrowing behavior of infected individuals have been attributed to the presence of the externa on the crab abdomen (Bishop and Cannon, 1979), which may hinder mobility and make escape from predators difficult. At the population level, the parasite–host relationship is highly unstable. Parasitic castration causes the reproductive death of the host crab, and so *L. panopaei* may act as an important regulator of host population density (Kuris, 1974). As the abundance of parasite larvae in the water column increases, mud crab abundance potentially decreases (Van Engel et al., 1966; Hines et al., 1997).

A decrease in mud crab abundance is likely problematic because these benthic crabs are important mesopredators within intertidal oyster reefs (Silliman et al., 2004) that regularly consume bivalves (e.g., eastern oyster *Crassostrea virginica* and the Atlantic ribbed mussel, *Geukensia demissa)* that act as bioengineers (McDermott, 1960; Seed, 1980; Bisker and Castagna, 1987). Thus these crabs are essential in energy flow throughout oyster reef habitats (Dame and Patten, 1981). A reduced mud crab population on intertidal oyster reefs may have implications for estuarine bivalves that provide essential ecosystem services, including water filtration, nutrient cycling, critical habitat for fish and invertebrates, and benthic-pelagic coupling (Coen and Lukenbach, 2000; Newell, 2004).

OBJECTIVES AND HYPOTHESES

This research explored prevalence and genetic relationships of *Loxothylacus panopaei* (Gissler, 1884) and its effects on the host *Eurypanopeus depressus* (Smith, 1869) in North and South Carolina. Objectives that include hypotheses, rationale and methodology are arranged by chapter.

CHAPTER 1:

There were two specific objectives in this chapter:

1. To determine if *L. panopaei* prevalence varied seasonally at three locations in northern South Carolina (Waties Island, Murrells Inlet and North Inlet). It was hypothesized there would be significant seasonal variation in *L. panopaei* prevalence because changes in seasonal water temperature are a major driver of larval distribution in an estuarine environment (Costlow and Bookhout, 1961). Walker et al. (1992) found starting at 25°C, *L. panopaei* larval broods are released every 5–6 days. Water temperatures in North Inlet, South Carolina reach and exceed 25°C at the beginning of June and do not drop below 25°C until the end of September (National Oceanic and Atmospheric Administration, Office of Ocean and Coastal Resource Management, NERRS System-wide Monitoring Program, 2004). To determine if parasite prevalence varied over time, monthly mud crab (*Eurypanopeus depressus* and *Panopeus herbstii*) collections were made from three locations in northern South Carolina and correlated with water temperature and salinity measurements taken at each collection.
2. To determine the source location of North and South Carolina *L. panopaei* populations, as these populations might originate from the northern (invasive) or

southern (native) populations. We hypothesized that *L. panopaei* of the Carolinas would be derived from the northern (invasive) populations. Kruse et al. (2012) found invasive *L. panopaei* in coastal Georgia, suspected to have travelled south from the Chesapeake Bay introduction ca. mid-1960s. DNA sequence analyses of the mitochondria-encoded cytochrome *c* oxidase subunit I locus (COI) were used to determine the source location of North and South Carolina *L. panopaei* populations.

CHAPTER 2:

The objective of this chapter was to investigate differences in foraging rates between parasitized and unparasitized *Eurypanopeus depressus*. We hypothesized that parasitized crabs would consume significantly fewer juvenile mussels than unparasitized crabs. Isaeva et al. (2005) found that bodies of parasitized hosts are weakened compared to unparasitized hosts, and so weakened crabs may display feeding behavior divergent from normal behavior. To determine differences in foraging rates, we compared the number of mussels consumed per unit time between externa-bearing and externa-lacking *E depressus*.

CHAPTER 3:

The purpose of this chapter was to examine relationships among host crab carapace width, and size and color of *L. panopaei* external reproductive structures. We hypothesized that *E. depressus* with larger carapace widths would exhibit larger *L. panopaei* externae. Reinhard and Reischman (1958) found the largest of the xanthid species studied in the native range, *Panopeus herbstii*, displayed larger externae than the smaller crab species. We also hypothesized that purple *L. panopaei* externae at the oldest developmental stage would be the largest. To examine these relationships,

the left-right axes of externae were measured and externae color was categorized following Wardle and Tirpak (1991). Infected *E. depressus* carapace width was also measured.

*This chapter is under review in the Journal of Parasitology (December 2013).

CHAPTER 1:

PREVALENCE OF THE INVASIVE RHIZOCEPHALAN PARASITE *LOXOTHYLACUS PANOPAEI* IN *EURYPANOPEUS DEPRESSUS* AND GENETIC RELATIONSHIPS OF THE PARASITE IN NORTH AND SOUTH CAROLINA

Introduction

Globalization has increased human-mediated dispersal of nonindigenous marine species over the past few centuries (Carlton, 1989; Cohen and Carlton, 1997), threatening local biodiversity and potentially causing species extinctions (Baskin, 1996; Wilcove et al., 1998). Terrestrial and aquatic species are transported across the globe by ships (Baskin, 1996) and become viable populations due to lack of competitors and predators (Elton, 1958; Crawley et al., 1986). There has been an increase in global transport of crustaceans including decapod crabs and barnacles via ships and their ballast water in recent decades (Cohen and Carlton, 1997; Kerckhof et al., 2010). The European green crab *Carcinus maenas* (Linnaeus, 1758) is native to the eastern Atlantic Ocean, but has been documented in both the northwest Atlantic and the eastern Pacific Oceans since 1989 (Cohen et al., 1995), and the Asian shore crab *Hemigrapsus sanguineus* (De Haan, 1835), native to the western Pacific Ocean, was found along the coast of New Jersey in 1988 (McDermott, 1991). *Petrolisthes armatus* (Gibbes, 1850), the green porcelain crab, is native to Brazil but was discovered on oyster reefs in Cape Canaveral, Florida in 1994 and has since spread north to North Inlet, South Carolina (Knott et al., 1999; Hollebone

and Hay, 2007). The titan barnacle *Megabalanus coccopoma* (Darwin, 1854), native to tropical eastern Pacific was recorded from the northern Gulf of Mexico, Georgia and the Carolinas in the early 2000s (Yamaguchi, et al., 2009). In addition to their ecologically disruptive effects, such invasive crustaceans harbor diseases and parasites that may cause harm to native biota (Bower et al., 1994; Cohen et al., 1995).

Loxothylacus panopaei (Gissler, 1884) is an invasive, parasitic barnacle now widespread on the US Atlantic coast that infects xanthid crabs and causes host castration (Walker, 2001; Kruse and Hare, 2007; Kruse et al., 2012; Freeman et al., 2013). Gulf of Mexico oysters were transported to the Chesapeake Bay area in the 1960s (Van Engel et al., 1966) to augment native populations that had declined because of overfishing and parasitic diseases such as Dermo and MSX (Van Engel et al., 1966; Bower et al., 1994; Rothschild et al., 1994). Mud crabs infected with the rhizocephalan parasite *L. panopaei* were also transported to Chesapeake Bay with these oysters where the parasite established populations in this new habitat (Van Engel et al., 1966).

Loxothylacus panopaei's native range extends from Cape Canaveral through south Florida into the Gulf of Mexico and as far east in Caribbean waters as Venezuela (Kruse and Hare, 2007; Kruse et al., 2012). It has invaded Atlantic coastal estuarine habitats from Long Island Sound in New York to just north of Cape Canaveral, Florida, (Hines et al., 1997; Kruse et al., 2012; Freeman et al., 2013), with prevalence ranging from 10–93% in the flatback mud crab *Eurypanopeus depressus* (Smith, 1869, e.g., Daugherty 1969; Kruse and Hare 2007; Kruse et al., 2012; O'Shaughnessy et al., in review).

This parasitic rhizocephalan barnacle has an extremely modified life cycle compared to free-living barnacles. The *L. panopaei* naupliar and cyprid larval stages are its only

morphological links to the Cirripedia (Høeg, 1995), while the adult form is unique and consists of an external reproductive sac (externa) and an internal rootlet system (interna) used for nutrient up-take and digestion (Høeg and Lützen, 1995). The rhizocephalan genus *Loxothylacus* also infects commercially valuable species such as the blue crab *Callinectes sapidus* Rathbun, 1896 (Bower et al., 1994) and the golden king crab *Lithodes aequispinus* Benedict, 1895 (Isaeva et al., 2005), which may be costly to US fisheries (Guillory et al., 1998).

Loxothylacus panopaei infection causes adverse effects for the host at the individual and population levels. Crabs are castrated by cessation of gonad maturation, with a subsequent inability to reproduce (Walker, 2001), effectively removing the crab from the genetic pool. Host ecdysis is also interrupted, halting growth of the infected individual (O'Brien and Skinner, 1990), and reduced feeding in parasitized *E. depressus* has been reported (O'Shaughnessy et al., in review). The crab is parasitized for the remainder of its life during which the parasite exploits the individual for food and reproduction (Isaeva et al., 2005).

Past studies have examined genetic diversity within the invasive range and traced the parasite's range expansion (Kruse and Hare, 2007; Kruse et al., 2012). DNA sequence analyses of the mitochondria-encoded cytochrome *c* oxidase subunit I locus (COI) was used to show that two distinct *L. panopaei* clades were present from Chesapeake Bay to Florida and into the Gulf of Mexico (Kruse et al. 2012): The 'ER clade' infecting only *E. depressus* and *Rhithropanopeus harrisii* (Gould, 1841), and the 'P clade' infecting mostly *Panopeus* crabs. The 'ER clade' consisted of hosts from both native and invasive ranges, while *L. panopaei* specimens in the 'P clade' were found exclusively on crabs in the

native range. Analysis of the nuclear cytochrome *c* (CtyC) showed three distinct clades along the same area: The 'ER clade' was consistent with results from COI analyses, while the 'P1 clade' and the 'P2 clades' infected *Panopeus* species exclusively (Kruse et al., 2012). These studies provided resolution to the question of host specificity and presence of distinct *L. panopaei* lineages.

Vectors controlling the southern *L. panopaei* range expansion from the original Chesapeake Bay population have not been identified. Infection is dependent on larval dispersal, and because the parasite's larval stages last approximately six days, water currents have been suggested as possible vectors within estuaries only. But the parasite has spread across disjointed estuaries and past potential barriers such as the Atlantic Intracoastal Waterway in Florida (Kruse et al., 2012). *Loxothylacus panopaei*-infected mud crabs have been observed in association with bryozoans and bivalves fouling large ships (Davidson et al., 2008), and so biofouling and ballast water exchange might be vectors of *L. panopaei* transport over larger distances.

Previous studies have examined the distribution, origin of introduction and host specificity of *L. panopaei* along the US Atlantic coast from Chesapeake Bay to southern Florida (Kruse and Hare, 2007; Kruse et al., 2012), however, North and South Carolina parasite populations were not investigated. To confirm that the range expansion of *L. panopaei* was continuous from Chesapeake Bay to northern Florida, the current study investigated North and South Carolina *E. depressus* populations for the presence of *L. panopaei*, and specific haplotypes of the parasite were identified using molecular analyses. To further investigate *L. panopaei* populations from the undocumented South Carolina parasite range, variation in monthly parasite prevalence was quantified at three

locations (Waties Island, Murrells Inlet and North Inlet) over a 13-month period (January 2012–January 2013), and host size and sex characteristics were examined.

Materials and Methods

Sampling

North Carolina: *Eurypanopeus depressus* mud crabs infected with *Loxothylacus panopaei* were collected by hand from intertidal oyster reefs (Hines et al., 1997) at three sites in North Carolina for DNA analysis (Appendix, Figure 1). Two of these sites were located within the Masonboro Island and Rachel Carson National Estuarine Research Reserves, respectively. The Masonboro Island site is a fringing oyster reef in Loosins Creek, New Hanover County (34°10′21″N, 77°49′57″W), adjacent to the Atlantic Intracoastal Waterway. Infected crabs were collected from a fringing oyster reef in the Rachel Carson Reserve along the Taylor Creek Channel (34°42′46″N, 76°40′23″W) adjacent to the Duke University Marine Laboratory in Carteret County. The third collection site was a small jetty in Bogue Sound (34°43′35″N, 76°49′15″W), west of Morehead City, Carteret County.

South Carolina: Monthly collections (January 2012–January 2013) of xanthid crabs were made by hand (Hines et al., 1997) from oyster reefs in Dunn Sound at Waties Island (33°51′11″N, 78°35′37″W), Murrells Inlet at the Garden City Causeway (33°34′45″N, 79°00′14″W), and Clambank Creek in the North Inlet-Winyah Bay National Estuarine Research Reserve (33°20′04″N, 79°11′33″W; Appendix, Figure 2). Sites included a patch

oyster reef of 875 m² in Dunn Sound, a patch reef of 240 m² at Garden City Causeway, and a thin stretch of fringing reef approximately 575 m long in Clambank Creek.

A collection was made by excavating all surface oyster clusters, buried shell and aerobic sediment from a 0.25 m^2 area. The excavated material was placed into a bin and clusters were broken apart by hand to ensure that xanthid crabs of all sizes were captured. This process was repeated until approximately 100 xanthid crabs were collected. Between December–March, mud crabs are less dense in the intertidal oyster reef (Dame and Vernburg, 1982), and so the target collection was reduced to 50 crabs. Each collection started from an adjacent undisturbed area on the reef, and subsequent 0.25 m^2 areas on a collection date were sampled every 3 m along the lower intertidal zone parallel to shore.

All crabs were placed into plastic containers and transported to the laboratory where they were frozen at or below 0°C until measurement and examination. Water temperature (°C) and salinity (parts per thousand, ppt) readings were taken at each collection with a YSI 30 water temperature and salinity meter (YSI, Inc. Yellow Springs, Ohio). In the laboratory, mud crabs were identified to species and sexed by external morphology (Williams, 1984). The abdominal flap of each crab was separated from the body and examined for the presence of an externa using a dissecting microscope. Crabs were classified as parasitized if an externa of any size was present (virgin or mature). Maximum carapace width (CW) of all crabs was measured to the nearest 0.1 mm using a digital caliper.

Genotyping

Fifty-seven *Loxothylacus panopaei* from North (n = 29) and South Carolina (n = 28) were selected for DNA analysis. All parasites analyzed were from *Eurypanopeus depressus* hosts. Approximately 20 mg of *L. panopaei* tissue was removed from the externa and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA), following the protocol for animal tissue. A portion of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was amplified by polymerase chain reaction (PCR) using primers that Kruse and Hare (2007) modified from Folmer et al. (1994). The modified COI primers that were used in this study were: Lxpa-L, 5′-GAGCAAGATTAATTGGAGGAGGT-3′ and Lxpa-R, 5′-GCCCCAGCTAAAACTGGTAA-3′ (Kruse and Hare, 2007). Amplification products were generated using Platinum Taq (Invitrogen, Grand Island, NY) or MyTaq (Bioline, Taunton, MA) DNA polymerases. The most consistent COI amplifications resulted from the following reaction conditions: 5.0 µl 5x MyTaq Red Reaction Buffer, 0.25 µl MyTaq HS DNA polymerase, 1 µl DNA template and 10 µM of each primer in a 25-µl reaction using the standard MyTaq PCR cycling conditions, but with an initial denaturation time of 2:45 and a 45°C annealing temperature.

Amplification products were cleaned using ExoSAP-IT (Affymetrix, Cleveland, OH), used as templates in BigDye v. 3.1 (Applied Biosystems, Foster City, CA) sequence reactions, and run on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems) in the DNA Analysis Core Facility at the Center for Marine Sciences, University of North Carolina Wilmington. Results of forward and reverse sequence reactions were assembled and edited using Sequencher (Gene Codes Corp., Ann Arbor, MI).

Analyses

South Carolina collections: Prevalence was calculated as the number of *Eurypanopeus depressus* with an externa divided by the total *E. depressus* collected, and expressed as a percentage with 95% confidence intervals. Monthly South Carolina prevalence data were normally distributed (Shapiro-Wilk), so traditional analysis of variance (ANOVA) was performed to determine if prevalences differed significantly across locations, and a Bonferroni pairwise comparison was used to compare each location prevalence to the others. The relationships between water temperature and prevalence, and between salinity and prevalence were examined using Pearson product-moment correlations. Carapace width between the total (infected and uninfected) female and male *E. depressus* collection was analyzed using a Student's *t*-test. Because infected crab CW data violated parametric assumptions (*i.e.*, were not normally distributed), a nonparametric Mann-Whitney-U test was used to compare CW between infected and uninfected *E. depressus*, and a Kruskal-Wallis test, with adjustment for multiple comparisons, was used to analyze CW differences among Waties Island, Murrells Inlet and North Inlet *E. depressus*. A Chi-squared test was used to assess if there was a significant difference in infection between female and male *E. depressus*. Tests were conducted in SigmaPlot v.12.0 (Systat Software, Inc., San Jose, CA) unless otherwise noted, and $\alpha = 0.05$.

Genetic: Twelve representative COI *Loxothylacus panopaei* (ingroup) and one COI *Loxothylacus texanus* Boschma, 1933 (outgroup) sequences generated by Kruse et al. (2012) were aligned with the 57 COI sequences from this study using MacClade v 4 (Maddison and Maddison, 2000) and MEGA v 5.1 (Tamura et al., 2011). Sequences from

this study were collapsed into representative haplotypes (n = 4) and their similarity to the Kruse et al. (2012) haplotypes assessed with UPGMA clustering using MEGA.

Population differentiation among the 126 native and invasive range specimens sequenced in Kruse et al. (2012) and this study was examined using an exact test of population differentiation (Raymond and Rousset, 1995) and a hierarchical analysis of molecular variance (AMOVA, Excoffier et al., 1992) as implemented in Arlequin (v. 3.1, Excoffier et al, 2005). Sampled sites were treated as "populations" in these analyses and these populations grouped into the following "regions": Chesapeake Bay; North Carolina to north Florida; south Florida Atlantic coast; Florida Gulf coast; and Louisiana Gulf coast. A minimum spanning tree was created using HapStar v. 0.7 (Teacher and Griffiths, 2011) to view connection distances among haplotypes.

Results

South Carolina parasite prevalence

A total of 2,373 *Eurypanopeus depressus* and *Panopeus herbstii* were collected from three oyster reef locations in northern South Carolina from January 2012–January 2013. More *E. depressus* (n = 1,615) were collected than *P. herbstii* (n = 731). Twenty-seven crabs could not be identified, and excluded from subsequent analyses, because they were too small, damaged or had recently molted. While all individuals were examined for the presence of the parasitic externa, only *E. depressus* were infected. *Loxothylacus panopaei* externae were found on 391 *E. depressus* crabs, for a total parasite prevalence of 24.2% across all three sites, ranging in monthly prevalence from 8.7–51.6% (Table 1). Mean

monthly prevalence of *L. panopaei* at Waties Island was 31.8% ± 6.9 (mean ± 95% CI), 19.3% ± 4.6 at Murrells Inlet, and 18.5% ± 4.2 at North Inlet (Figure 1). Parasite prevalence was significantly different between Waties Island and North Inlet (Bonferroni pairwise comparisons, $t = 3.8$, $p = 0.001$), and between Waties Island and Murrells Inlet ($t = 3.6$, $p = 0.003$), but not between Murrells Inlet and North Inlet ($t = 0.3$, $p = 0.966$).

Loxothylacus panopaei was found during every monthly collection at water temperatures and salinities ranging from 9.4–34.6°C and 24.5–39.7 ppt, respectively. Monthly parasite prevalence was not significantly correlated with spot water temperature taken at each location from January 2012–January 2013 (Pearson product moment correlation; Waties Island, $r = 0.32$, $p = 0.29$; Murrells Inlet, $r = 0.32$, $p = 0.28$; North Inlet, $r = 0.05$, $p = 0.87$). However, prevalence increased with increasing water temperature from January 2012–April 2012 at Waties Island, and from January 2012–July 2012 at North Inlet; Appendix, Figure 3). Prevalence at Murrells Inlet did not correlate with water temperature during any part of the year. Parasite prevalence and salinity were not significantly correlated at Waties Island ($r = 0.17$, $p = 0.57$) and Murrells Inlet ($r = 0.16$, $p = 0.60$), but there was a significant correlative negative relationship between prevalence and salinity at North Inlet ($r = -0.59$, $p = 0.03$). Prevalence was the highest on 1 May 2012 at Waties Island (51.6%), on 29 May 2012 at Murrells Inlet (36.6%) and on 26 January 2013 at North Inlet (28.6%), although on 29 June 2012, prevalence was also relatively high at North Inlet (27.3%).

Size and sex characteristics of South Carolina **Eurypanopeus depressus**

The total (infected and uninfected, n = 1,615) mean CW of the *E. depressus* collection among the three South Carolina sites was 8.6 mm ± 3.0 (mean ± SD), with mean CW of females (8.4 mm ± 2.6, n = 1,008) significantly smaller than mean CW of males (9.0 mm ± 3.5, n = 600; Student's *t*-test; $t = -3.81$, $d.f. = 1606$, $p < 0.001$). Female *E. depressus* were significantly smaller than male *E. depressus* at Waties Island ($t = -2.48$, $d.f. = 565$, $p = 0.01$) and North Inlet ($t = -2.69$, $d.f. = 506$, $p = 0.01$), but not at Murrells Inlet ($t = -1.46$, $d.f = 531$, $p = 0.14$). The mean CW of infected *E. depressus* was 9.8 mm ± 1.5 with a range of 5.8–16.3 mm, while the uninfected *E. depressus* collection had a mean CW of 8.2 mm ± 3.2, ranging from 2.4–20.3 mm. The CW distribution of infected *E. depressus* was unimodal, with the most infected individuals in the 9.1–10.0 mm CW range (Figure 2). The CW of infected *E. depressus* was significantly larger than the CW of the total *E. depressus* collection (Mann-Whitney-U; $U(1) = 2.17 \times 10^5$, $z = -9.61$, $p < 0.001$). The mean CW of infected *E. depressus* at Waties Island was 9.7 mm ± 1.5, 10.0 mm ± 1.7 at Murrells Inlet and 9.7 mm ± 1.5 at North Inlet (Figure 3). The analysis of CW distribution in infected crabs showed no significant differences among the three locations (Kruskal-Wallis; $H = 0.84$, $p = 0.66$, $d.f. = 2$).

More female (n = 1,008) than male (n = 600) *E. depressus* were collected at all locations, and a higher percentage of females (26.8%) than males (19.9%) were infected (Figure 4). Seven *E. depressus* could not be sexed because of damage, small size or a recent molt. The sex ratio of the total (infected and uninfected) *E. depressus* collection was 1.7:1 (F:M), with sex ratios of 1.8:1, 1.5:1 and 1.7:1 at Waties Island, Murrells Inlet and North Inlet, respectively. The sex ratio of infected *E. depressus* was 2.3:1, with sex

ratios of 2.1:1, 2.3:1 and 2.7:1 at Waties Island, Murrells Inlet and North Inlet, respectively. Significantly more female than male *E. depressus* than expected were infected with *L. panopaei* across all locations (χ^2 (1, n = 391) = 7.6, p = 0.006). The expected ratio of female to male *E. depressus* in the infected individuals was calculated from the overall sex ratio of uninfected *E. depressus*.

North Carolina collection

Fifty-four infected *E. depressus* were collected from the three North Carolina locations. Twelve infected *E. depressus* were collected from Masonboro Island, 23 from Rachel Carson Reserve, and 19 from the Bogue Sound Jetty. One *E. depressus* at each location had two externae, for a total of 57 *L. panopaei* specimens collected from North Carolina. The mean CW of infected *E. depressus* was 11.4 mm ± 1.6 (mean ± SD) at Rachel Carson Reserve, 9.7 mm ± 1.3 at the Bogue Sound Jetty and 10.4 mm ± 1.8 on Masonboro Island. The total mean CW for all infected North Carolina *E. depressus* was 10.6 mm ± 1.7, and the total CW range was 7.5–15.2 mm.

Molecular analyses

A total of 57 *Loxothylacus panopaei* COI sequences from North and South Carolina were analyzed and compared to 12 *L. panopaei* (ingroup) and one *L. texanus* (outgroup) COI sequences generated from Kruse et al. (2012). No insertions or deletions were required to align sequences, and the 5′ and 3′ ends of the alignment were trimmed to remove sites with missing data. Among the *L. panopaei* sequences generated in this study, four sites were variable along the 424-base pair sequence alignment (0.94%). All

sequences from the current study with the exception of one were identical to COI Haplotypes 1, 2 and 3 identified by Kruse et al. (2012; Table 2; Figure 6). Haplotype 1 (H_1) and Haplotype 2 (H_2) are present in specimens exclusively from the invasive range, while Haplotype 3 (H_3) is present in specimens from both the native and invasive ranges of *L. panopaei*. A new haplotype was found in a single specimen from the Rachel Carson Reserve in Carteret County, North Carolina, and is referred to as H_{NC}. This sequence was one base pair different from the Chauvin12 haplotype (alignment site 384) and two base pairs different from the most frequently encountered H_3 (alignment sites 142 and 361; Figure 6). The *L. panopaei* sequenced in this study included 12 specimens with H_1, nine with H_2, 35 with H_3 and the one unique specimen, H_{NC}. All six collection locations had at least one representative from each haplotype H_1–H_3 with the exception of Waties Island where H_1 was absent (Figure 7).

The AMOVA found variation among "regions" and within "populations" or sites to be significant ($p < 0.0001$ for both) with the largest proportion of variation occurring within sites (67.7%). Exact tests of population differentiation showed that the variation among regions is based upon differences between the Fort Pierce, Florida and Panacea, Florida populations (both had a large proportion of sampled specimens with the divergent H_4) and the other populations.

Discussion

To our knowledge *Loxothylacus panopaei* had not been previously reported from Waties Island and Murrells Inlet, South Carolina, and Masonboro Island, North Carolina. In collections from Long Island Sound, New York, Freeman et al. (2013) found 11.9% *L.*

panopaei prevalence in *E. depressus*. In a Chesapeake Bay study that documented the early invasion of *L. panopaei* in the mid-Atlantic, mean monthly infection of *E. depressus* ranged from 2.6% ± 2.8 to 70.0% ± 29.0 (mean ± 95% CI; Daugherty, 1969). Collections of *E. depressus* from coastal Georgia recorded prevalence from 50–67% (Kruse and Hare, 2007), and studies of northern Florida parasite populations in *E. depressus* found prevalence from 2–93% (Kruse and Hare, 2007; Kruse et al., 2012). Consistent with prevalences reported from other locations in the parasite's invasive range, mean monthly prevalence from this study ranged from 18.5% ± 4.2 to 31.8% ± 6.9 (mean ± 95% CI), and was as high as 51.6% on Waties Island.

A previous search for *L. panopaei* along mid-Atlantic and southeastern Atlantic shores from the 1980s recorded occurrences in Chesapeake Bay (Maryland and Virginia), Bogue Sound in North Carolina, and south of Port Canaveral in Florida (Hines et al., 1997). The parasite was absent from South Carolina, Georgia, and north Florida collections. One of their South Carolina sample sites was North Inlet, South Carolina where the current study found a mean monthly prevalence of 18.5% ± 4.2 (mean ± 95% CI). The absence of *L. panopaei* in North Inlet in the Hines et al. (1997) study could be attributed to a small sample size (n = 20) of *E. depressus* hosts. Monthly sample sizes of *E. depressus* at North Inlet from this study were never less than 12, but the mean sample size was 39 ± 15 (mean ± SD). However, in December 2012 and January 2013—months with small sample sizes (n = 12 and n = 21, respectively)—*L. panopaei* prevalence was 25.0% and 28.6%, respectively. The relatively high prevalence reports from small sample sizes here suggest the parasite may have been absent from the North Inlet *E. depressus* population in 1986.

Although prevalence of *L. panopaei* was not significantly correlated with water temperature, trends from this study indicate that infected crabs are less abundant when water temperatures are lower on intertidal oyster reefs (Waties Island and North Inlet, but not Murrells Inlet). Two possible explanations exist for the seasonal decline in prevalence: (1) there may be a seasonal population movement of infected crabs from the intertidal to the subtidal zone (Daugherty, 1969), requiring a modification in collection methods; or (2) *Loxothylacus panopaei* does not replace individuals lost to predation in the cooler months because the parasite is not reproducing (reproduction occurs around 25°C; see Walker et al., 1992), and so prevalence declines. Predation on crabs with the parasitic externa might be higher than crabs without the externa because mobility is reduced with infection. Wardle and Tirpak (1991) found that blue crabs (*Callinectes sapidus*, Rathbun, 1896) infected with *L. texanus* rarely burrowed below the sediment, and sand crabs (*Portunus pelagicus*, (Linnaeus, 1758)) infected with the rhizocephalan *Sacculina granifera* Boschma, 1973 buried in sediment at a significantly slower rate than uninfected sand crabs (Bishop and Cannon, 1979).

There was a significantly larger proportion of female to male *E. depressus* infected in the current collection (2.3:1; χ^2 (1, n = 391) = 7.6, p = 0.006), similar to findings by Daugherty (1969) in Chesapeake Bay who found 20.6% of the males and 30.4% of the females with infection. *Loxothylacus panopaei* larvae have been shown to settle in the host branchial chamber within the 24 hour post-molt period (Walker et al., 1992), and so molting frequency is likely the determinant of infection. Female *E. depressus* reach maturity at 5.5–6.4 mm CW, while males mature at 5.1–6.0 mm CW (Ryan, 1956), suggesting females may have to molt at least once more before reaching terminal molt.

Molting frequency may not be the only determinant of infection. Daugherty (1969) suggested that infected male *E. depressus* might simply experience a higher mortality than infected females, and Høeg and Lützen (1995) maintained that the broad abdomen of a female host supports an externa better than the thinner male abdomen. Maturing female xanthids are equipped with pleopods long enough to extend beyond the tip of the abdomen (Hines 1989), which allow it to support the parasitic externa as if it was a brood sac.

In the current study, infected *E. depressus* had significantly larger CW than uninfected crabs. Past observations noted that crabs with rhizocephalan infection are generally smaller than crabs without infection (Van Engel et al., 1966; Høeg, 1995), which is supported by the occurrence of terminal anecdysis (Hartnoll 1965)—the cessation of molting at maturity—where the host gains a refuge from infection. However, Hines et al. (1997) found that almost the entire size range of their *E. depressus* collection (6–18 mm CW) was vulnerable to *L. panopaei* infection, and Alvarez et al. (1995) saw no significant difference in CW between infected and uninfected *E. depressus* in a laboratory infection study. The current study collected crabs indiscriminately in regards to size (size range of *E. depressus* collection was 2.4–20.3 mm CW). Other studies limited their collections to certain size ranges (i.e. O'Brien and Skinner (1990) collected only *E. depressus* 6–14 mm CW), which might cause the CW of the infected group to appear smaller than the uninfected group.

Consistent with *L. panopaei* population investigations in other parts of the invasive range, this study found infection in only *E. depressus* crabs. *Panopeus herbstii*—a mud crab that coexists with *E. depressus* in intertidal oyster reefs in South Carolina

(McDonald, 1982)—composed 45.3% of the monthly collections, but never exhibited the parasitic externa. Analyses of mitochondrial COI and nuclear CytC loci have shown that *L. panopaei* is composed of distinct lineages that are host-specific (Kruse et al., 2012), and that only in the parasite's native range is *P. herbstii* infected, confirming the absence of *L. panopaei* on *P. herbstii* hosts in this study.

Kruse et al. (2012) concluded that the source of *L. panopaei* infection in Chesapeake Bay was from Gulf of Mexico parasites in the 'ER clade', likely from west of the Mississippi River. Sequences from this study support this invasion pathway. H_1 and H_2 are composed of parasites found only in the invasive range (Chesapeake Bay–northern Florida), while H_3 consists of *L. panopaei* from both the invasive and native ranges (Kruse et al., 2012). The majority of sequences from this study (n = 35 of 57) fell into H_3, while H_1 and H_2 had 12 and 9 representatives, respectively. The previously unidentified H_{NC} is closest to a Chauvin, Louisiana sequence. We can therefore pose two hypotheses about the source of the North and South Carolina *L. panopaei* populations: (1) Some of the North and South Carolina parasites in H_3 and the single H_{NC} are a direct result of human-mediated translocation of infected crabs from the Gulf of Mexico to the Carolinas; or more likely, (2) these parasites represent a southern range expansion from the Chesapeake Bay 'ER clade' invasive population. The spread of invasive *L. panopaei* from Chesapeake Bay to North and South Carolina can likely be explained by vessel biofouling (Davidson et al., 2008) and movement of ballast water (Cohen and Carlton, 1997; Kerckhof et al., 2010) from one port (estuary) to another over large distances.

With the first documentation of *L. panopaei* at Waties Island and Murrells Inlet, South Carolina, this study fills a critical geographic gap between North Carolina and Georgia

parasite populations and existing prevalence reports. Currently, the invasive range of *L. panopaei* is continuous from Long Island Sound, New York, south to just north of Cape Canaveral, Florida. Mitochondrial COI locus analysis indicates that North and South Carolina *L. panopaei* populations are most probably a result of the Chesapeake Bay parasite population range expansion south, which continues through to northern Florida.

Table 1. Prevalence of *Loxothylacus panopaei* infection in *Eurypanopeus depressus* hosts from January 2012–January 2013 from three northern South Carolina locations. The last row indicates mean monthly parasite prevalence at each location with 95% confidence interval. The highest prevalence was from a collection on 1 May 2012 at Waties Island (51.6%). The number in parentheses represents the number of total *E. depressus* collected at that location over the 13 month period.

Waties Island		**Murrells Inlet**		**North Inlet**	
Date	% Prevalence (n = 568)	Date	% Prevalence (n = 536)	Date	% Prevalence (n = 511)
19 Jan 2012	16.0 (25)	2 Feb 2012	20.0 (30)	26 Jan 2012	9.5 (63)
23 Feb 2012	23.4 (47)	29 Feb 2012	17.9 (39)	28 Feb 2012	11.6 (43)
31 Mar 2012	37.9 (57)	30 Mar 2012	24.7 (77)	27 Mar 2012	14.8 (55)
1 May 2012	51.6 (63)	29 Apr 2012	14.6 (48)	30 Apr 2012	18.2 (44)
31 May 2012	32.6 (46)	29 May 2012	36.6 (41)	30 May 2012	26.4 (52)
28 Jun 2012	49.3 (70)	26 Jun 2012	23.9 (46)	29 Jun 2012	27.2 (33)
28 Jul 2012	40.0 (45)	30 Jul 2012	10.5 (56)	31 Jul 2012	21.9 (30)
1 Sep 2012	31.0 (58)	30 Aug 2012	14.9 (67)	28 Aug 2012	13.8 (58)
29 Sep 2012	14.3 (21)	26 Sep 2012	20.7 (29)	1 Oct 2012	13.3 (30)
30 Oct 2012	33.3 (30)	29 Oct 2012	25.8 (31)	31 Oct 2012	20.6 (34)
28 Nov 2012	20.0 (50)	27 Nov 2012	11.1 (27)	29 Nov 2012	8.8 (34)
1 Jan 2013	32.1 (28)	3 Jan 2013	8.7 (23)	2 Jan 2013	25.0 (12)
26 Jan 2013	32.1 (28)	27 Jan 2013	21.7 (22)	28 Jan 2013	28.6 (23)
Mean prevalence	31.8 ± 6.9		19.3 ± 4.6		18.5 ± 4.2

Table 2. Representative specimen ID and haplotype designations (Kruse et al., 2012), representative specimen collection location, number of NC/SC specimens sharing haplotypes, and GenBank accession numbers for *Loxothylacus panopaei* haplotypes found in this study.

Specimen ID	Collection location	Number with haplotype	Accession number
20120529_MI_1 (H_1)	Murrells Inlet, SC	12	KF530191
20120530_NI_11 (H_2)	North Inlet, SC	9	KF530192
20121128_WI_2 (H_3)	Waties Island, SC	35	KF530193
20120621_RCR_5 (H_{NC})	Rachel Carson Reserve, NC	1	KF530194

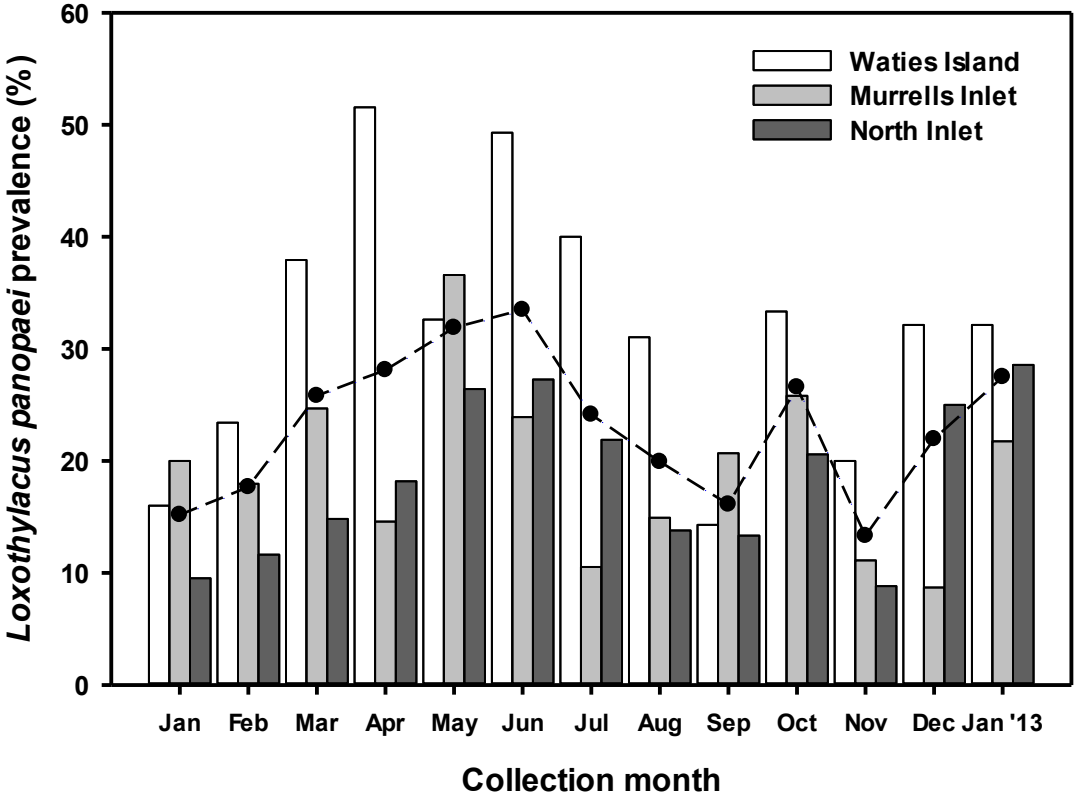


Figure 1. Monthly prevalence of *Loxothylacus panopaei* was significantly higher at Waties Island (31.8% ± 6.9; mean ± 95% CI) than at Murrells Inlet (19.3% ± 4.6) and at North Inlet (18.5% ± 4.2), South Carolina from January 2012–January 2013. The highest prevalence was recorded from the April collection at Waties Island (51.6%). The dashed line represents the mean parasite prevalence among the three sites each month.

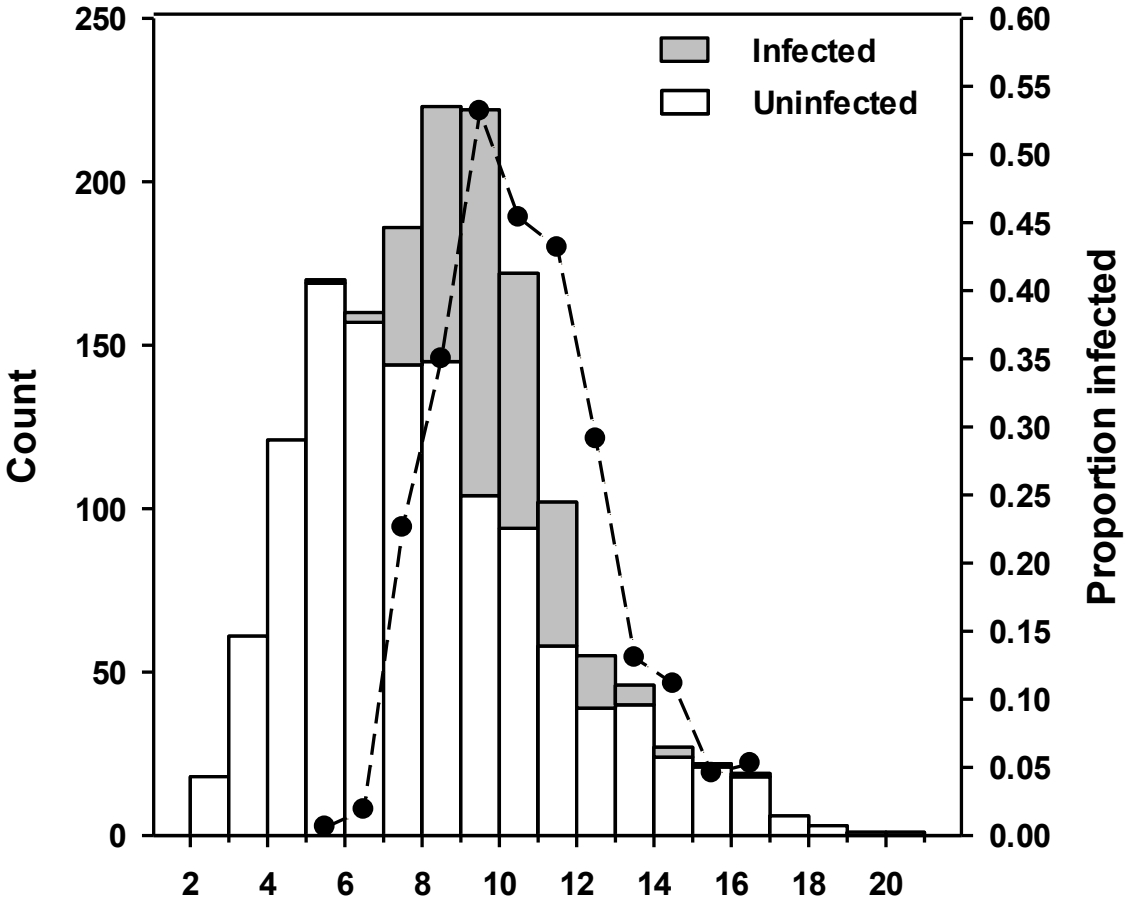


Figure 2. Carapace width (mm) of South Carolina *Eurypanopeus depressus*, including infected (externa-bearing; n = 391) and uninfected *E. depressus* (n = 1,224) from January 2012–January 2013. CW range was broken up into 19 1-mm bins ranging from 2–20 mm. CW range of infected crabs was 5.8–16.3 mm, with the 9.1–10.0 mm bin containing the most infected individuals (n = 118). CW range of uninfected crabs was 2.4–20.3 mm, with the 5.1–6.0 mm bin containing the most uninfected individuals (n = 169). The dashed line indicates the proportion of *E. depressus* that were infected in that size range.

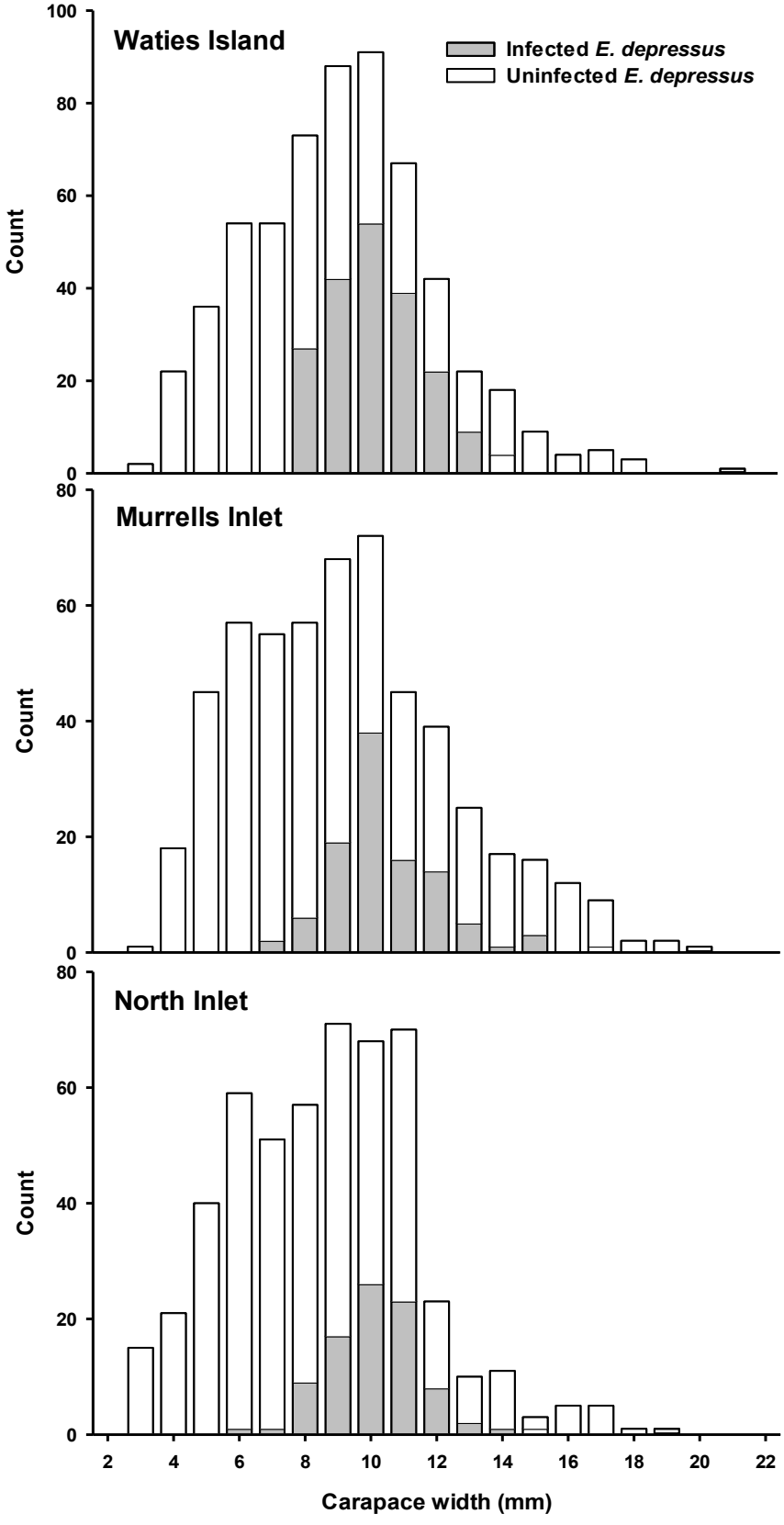


Figure 3. CW frequency (mm) of *Loxothylacus panopaei* infection in *Eurypanopeus depressus* at Waties Island, Murrells Inlet and North Inlet, South Carolina. There were no significant differences in infected crab carapace width among the three locations (p = 0.66).

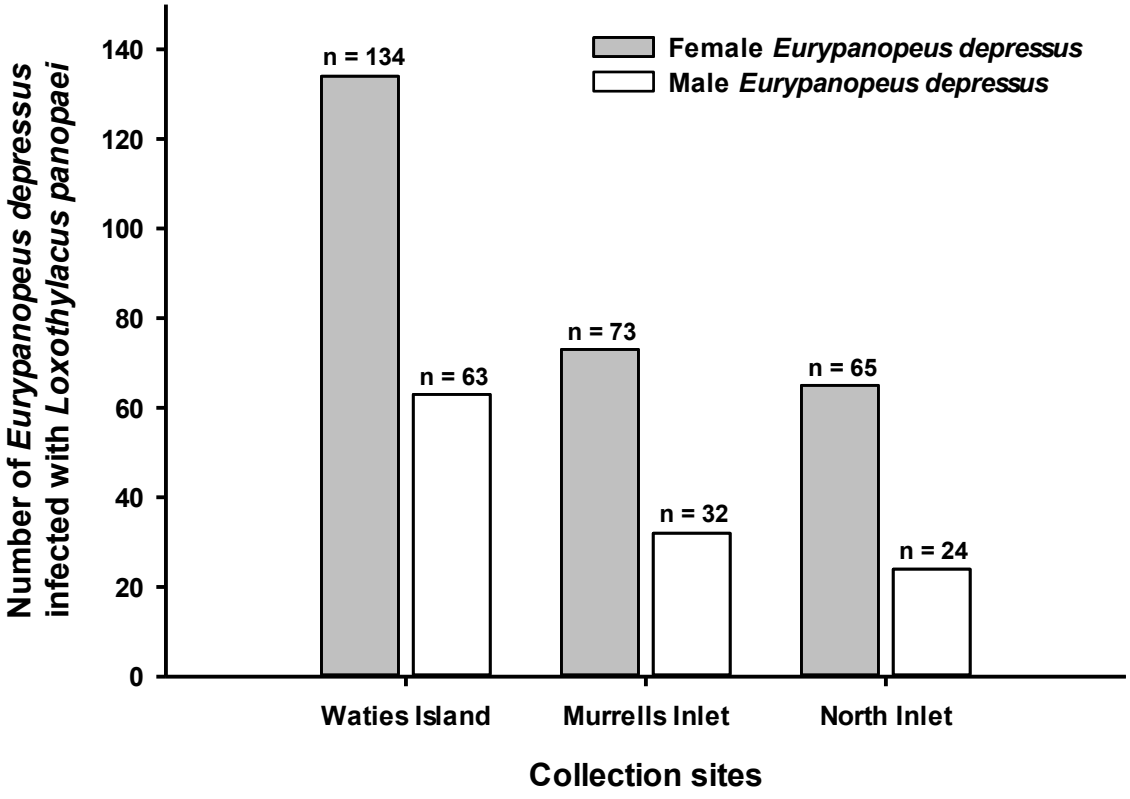


Figure 4. Significantly more female than male *Eurypanopeus depressus* than expected were infected with *Loxothylacus panopaei* across all locations (p = 0.006), but there was no significant difference at each individual location. The sex ratio of infected *E. depressus* among all locations was 2.3:1, with sex ratios of 2.1:1, 2.3:1 and 2.7:1 at Waties Island, Murrells Inlet and North Inlet, respectively.

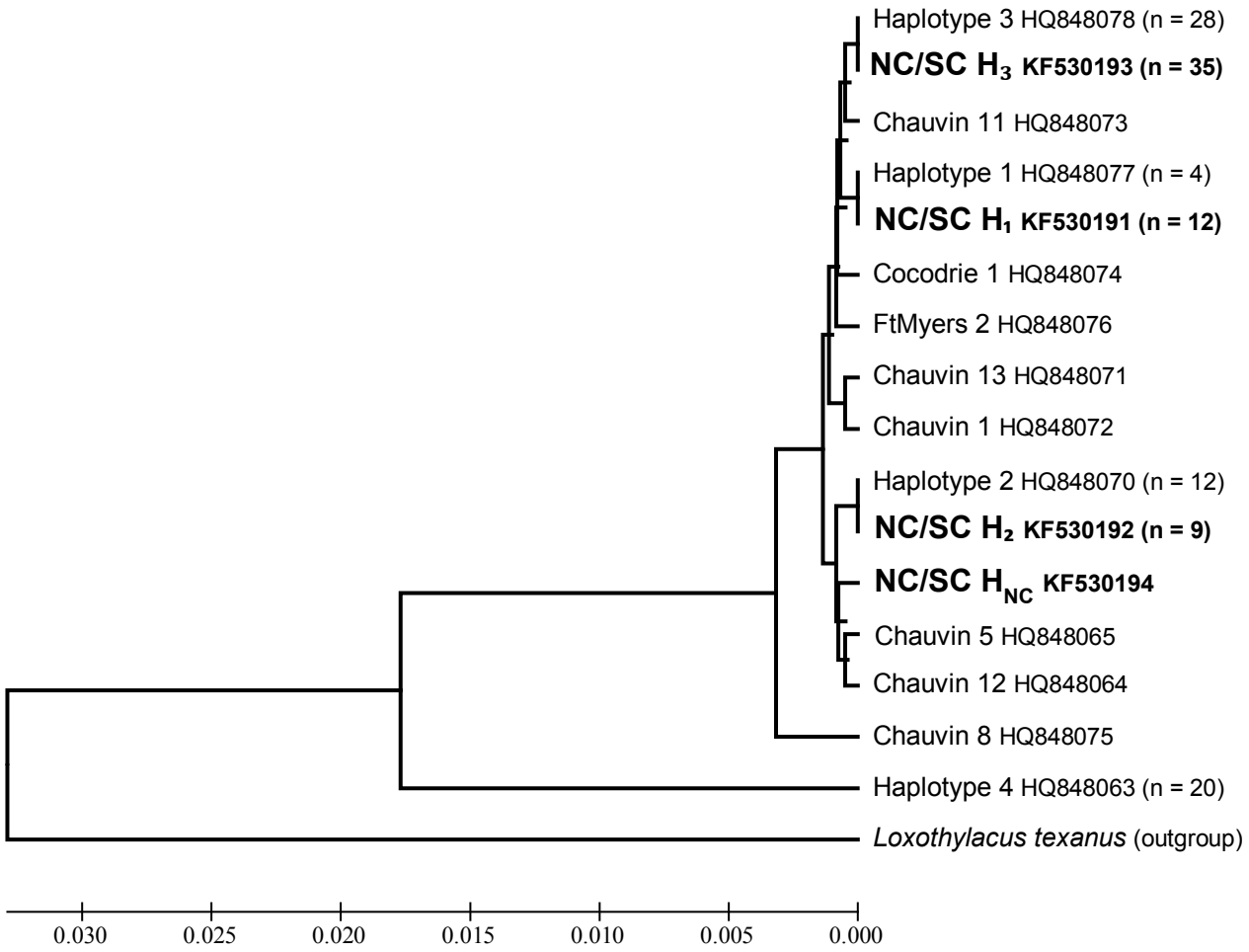


Figure 5. UPGMA cluster diagram of COI haplotypes from *Loxothylacus panopaei* (ingroup) and *Loxothylacus texanus* (outgroup) specimens. Haplotype designations for previously published sequences follow Kruse et al. (2012). GenBank accession numbers and the number of specimens sharing identical haplotypes are given for each. Sequences from North Carolina (NC) and South Carolina (SC) specimens from this study are highlighted.

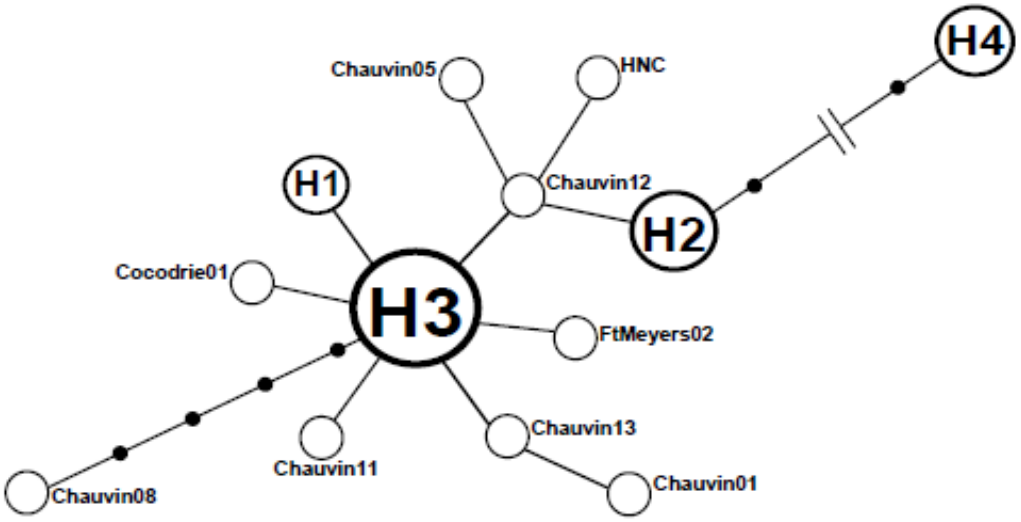


Figure 6. Minimum spanning tree of *Loxothylacus panopaei* COI haplotypes. Lines connecting circles indicate single base pair differences and black dots represent hypothetical haplotypes not found in the sampled specimens. Haplotype designations follow Kruse et al. (2012) and those found in multiple specimens are indicated by larger circles (H_1 = 16; H_2 = 21; H_3 = 63; H_4 = 20). The distance between H_2 and H_4 equals 33 base pair differences.

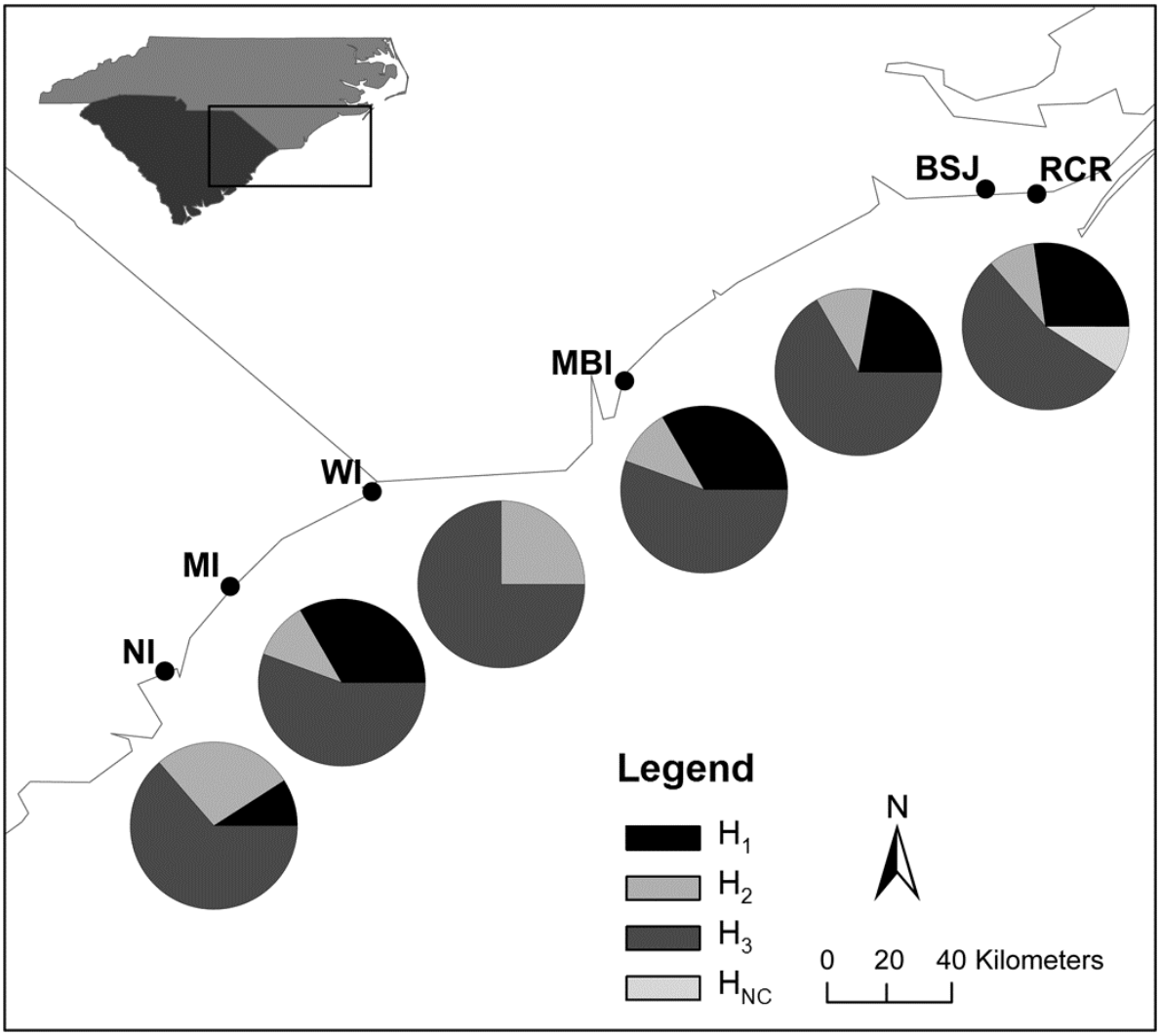


Figure 7. Distribution of *Loxothylacus panopaei* COI haplotypes (n = 57) at six locations: RCR = Rachel Carson Reserve, NC; BSJ = Bogue Sound Jetty, NC; MBI = Masonboro Island, NC; WI = Waties Island, SC; MI = Murrells Inlet, SC; NI = North Inlet, SC. Haplotype 3 was most frequent (n = 35) while Haplotype NC was least frequent (n = 1).

*A portion of Chapter 2 is accepted for publication in the Bulletin of Marine Science following minor revisions (December 2013).

CHAPTER 2:

REDUCED ECOLOGICAL FUNCTIONING OF THE FLATBACK MUD CRAB *EURYPANOPEUS DEPRESSUS* BY INFECTION WITH THE INVASIVE PARASITIC RHIZOCEPHALAN BARNACLE *LOXOTHYLACUS PANOPAEI*

Introduction

The Rhizocephala is comprised of parasitic barnacles that castrate decapod crustaceans, including xanthid crabs. Parasitic anecdysis of the crab host results from infection (O'Brien and Van Wyk, 1985), while endocrine and central nervous systems sustain damage from the parasitic internal rootlet system (Høeg, 1995). This internal system ramifies throughout the host hemolymph, absorbing nutrients, and emerges from the crab abdomen as a reproductive sac called the externa (O'Brien and Van Wyk, 1985). The rhizocephalan barnacle *Loxothylacus panopaei* (Gissler, 1884) infects intertidal mud crab species (Daugherty, 1969), transforming the abdomen of infected males into the broad abdomen of females, and inhibiting females from producing eggs (Høeg, 1995).

Loxothylacus panopaei is native in coastal estuarine habitats from Cape Canaveral, Florida south into the Gulf of Mexico and into Caribbean waters (Hines et al., 1997; Kruse et al., 2012). Crabs infected with *L. panopaei* were transplanted from the Gulf of Mexico to Chesapeake Bay with oysters during the mid-1960s (Van Engel et al., 1966). Since then *L. panopaei* has invaded western Atlantic habitats from Long Island Sound,

New York to just north of Cape Canaveral, Florida (Kruse and Hare, 2007; Kruse et al., 2012; Freeman et al., 2013).

Infection with *L. panopaei* adversely affects individual mud crabs. Reproduction and growth are halted (O'Brien and Van Wyk, 1985), and the host's ability to compete with conspecifics may be reduced (Daugherty, 1969). Crab feeding behavior may be compromised by internal damage to their organs from the parasitic rootlet system (Høeg, 1995) and the presence of the parasitic externa (Bishop and Cannon, 1979). Parasitic castration removes the infected individual from the genetic pool, and potentially lowers the effective population size (Van Engel et al., 1966; Daugherty, 1969).

Mud crabs exert top-down control on the eastern oyster *Crassostrea virginica* (Gmelin, 1791; e.g., McDermott, 1960; Bisker and Castagna, 1987) and the Atlantic ribbed mussel, *Geukensia demissa* (Dillwyn, 1817; e.g., Seed, 1980) within temperate intertidal oyster reefs (Silliman et al., 2004). In intertidal oyster reef habitats along the US Atlantic coast, *Eurypanopeus depressus* (Smith, 1869) feeds on small bivalves (McDermott, 1960; Kulp et al., 2011) and macroalgae in the oyster cultch interstices (Meyer, 1994). Almost 40% of *C. virginica* (5.9 mm mean shell length) made available to *E. depressus* crabs (15.8 mm mean carapace width, CW) were consumed over 96 hours at 25°C (Kulp et al., 2011), and McDermott (1960) observed *E. depressus* consumption of *C. virginica* (3–30 mm) at a rate of 1.6 oysters 24 hours^{-1} crab^{-1} at 23°C.

Community-level changes in intertidal oyster reef trophic structure may occur when parasites are prevalent (Mouritsen and Poulin, 2002). Because *L. panopaei* is a new parasite to the US Atlantic coast, the effects on native host populations and related trophic structure are unknown but likely negative (Lafferty and Kuris, 1996; Ruiz et al.,

1997). The presence of *L. panopaei* on an oyster reef may impact predator (*E. depressus*) demographics, population size and, thus, the relative importance of prey (bivalve) species.

Prior to this study, there was only one published investigation of *L. panopaei* in South Carolina waters (Hines et al., 1997). Recent parasite prevalence studies have been restricted mainly to Florida (Tolley et al., 2006; Kruse and Hare, 2007; Kruse et al., 2012), Georgia (Hines et al. 1997, Kruse and Hare 2007, Kruse et al. 2012), North Carolina (Reisser and Forward 1991, Hines et al. 1997), Virginia and Maryland (Van Engel et al., 1966; Daugherty, 1969), and New York (Freeman et al., 2013). Mud crab feeding rates have been experimentally determined (Seed, 1980; Milke and Kennedy, 2001; Kulp et al., 2011), but feeding behavior in mud crabs parasitized by *L. panopaei* has not been examined, and little is known about the overall effects of the Rhizocephala on food webs in intertidal oyster reef ecosystems. The purpose of this study was to examine prey consumption in *E. depressus* infected with *L. panopaei* under laboratory conditions.

Materials and Methods

Laboratory experiments were conducted in May and June 2012 in flow-through seawater tanks at the University of South Carolina Baruch Marine Field Laboratory, located in the North Inlet-Winyah Bay National Estuarine Research Reserve near Georgetown, South Carolina. Experimental mud crabs (*E. depressus*; 8–13 mm CW) were collected by hand from oyster clusters in Clambank Creek, North Inlet, (33°20′04″N, 79°11′33″W), an ocean-dominated tidal creek with a mean tidal range of

1.7 m (Dame et al., 1986). Mussels (*G. demissa* and *Brachidontes exustus*; 5–9 mm shell length) were collected from pilings and among oyster clusters in Clambank Creek.

Experimental *E. depressus* were given at least 72 hours to acclimate to laboratory conditions, and were maintained on natural, living oyster clusters in a flow-through seawater tank adjacent to and receiving the identical flow rate (19 L min^{-1}) as the experimental tanks. Mussels were collected one week before experimental runs, acclimated to laboratory conditions, and allowed to attach via byssal threads to matte ceramic tiles (95 × 95 mm), before placement in experimental containers. Source water was pumped from Oyster Landing in Crab Haul Creek, North Inlet (33° 20'57"N, 79°11'20"W). Crab sex and species were assessed in the field and confirmed with a dissecting microscope post experiments to minimize handling stress prior to experiments.

Two treatments were used: unparasitized and parasitized *E. depressus*. The unparasitized treatment consisted of male *E. depressus* mud crabs (8–13 mm CW). Male *E. depressus* were used to avoid accidental inclusion of gravid females that feed at lower frequencies than nongravid crabs (Mantelatto and Christofoletti, 2001). Externa-bearing *E. depressus* (8–13 mm CW) of both sexes were used as the parasitized treatment because parasitic castration effects made sex difficult to discern in the field (Daugherty, 1969). Crabs 8–13 mm CW were used because concurrent field collections in Clambank Creek found that this size range accounted for 90% (52 of 58) of parasitized crabs.

Crabs were arbitrarily assigned to identical containers (150 × 150 × 90 mm) and separated into treatment-specific tanks to prevent the spread of parasite larvae to the unparasitized treatment. Containers held 0.75 L of seawater and had approximately 60 holes 3 mm in diameter throughout to promote seawater exchange. Crabs were starved

for 12 hours prior to the start of each experiment to standardize hunger levels (Bisker and Castagna, 1987). Experiments were conducted in two flow through seawater tanks (100 cm diameter and 40 cm height), each of which held >17 experimental containers depending on how many crabs were available for each treatment after collection and acclimation period. Treatments were switched between tanks at the start of each experiment to minimize potential tank effects. The experiment was conducted four times, with each experiment containing 19–23 parasitized and 17–21 unparasitized crabs housed individually in containers. A single experiment lasted 72 hours and consisted of identical containers, each containing one crab of either treatment and 15 live mussels attached to a tile.

Flow-through seawater temperature (23.9°C ± 2.0, mean ± SD) and salinity (32.8 ppt ± 2.7) were measured twice daily during the experiments. Daily mean water temperature and salinity in Clambank Creek when experimental crabs and mussels were collected (2 May–2 June 2012) were 23.7°C ± 1.2 and 34.6 ± 2.3, respectively (National Oceanic and Atmospheric Administration, Office of Ocean and Coastal Resource Management, NERRS System-wide Monitoring Program, 2004). The target temperature and salinity conditions for this study were 20–25°C and 24–35 ppt, respectively (Whetstone and Eversole, 1981; Bisker and Castagna, 1987).

Any mussels remaining in containers at the end of the 72 hour period were counted and consumption rate was estimated as the number of mussels eaten $crab^{-1}$ 72 $hours^{-1}$. Normal feeding behavior in crabs is disrupted immediately prior to ecdysis (O'Halloran and O'Dor 1988), so containers were examined at the conclusion of each experiment for the presence of molted exoskeletons and soft crabs. All *E. depressus* were frozen at the

conclusion of each experiment for subsequent examination of sex and measurement of CW. Because the time from parasite settlement on the host to inoculation takes 48–72 hours, and from infection to externa emergence (internal phase) is 25–42 days (Walker et al., 1992; Glenner, 2001), some *E. depressus* in the unparasitized treatment may have been infected but not yet have expressed an externa. Crabs in the unparasitized treatment were therefore opened, microscopically examined, and tissue (crab and parasite) was extracted for DNA using DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA), following the protocol for animal tissue. Extracted DNA was amplified (PCR) using *L. panopaei*-specific primers to identify the presence of the interna stage (Sherman et al., 2008). The PCR conditions used to amplify the parasite and host 18S rDNA were: 5.0 µl 5x MyTaq Red Reaction Buffer, 0.25 µl MyTaq HS DNA polymerase, 1 µl DNA template and 10 µM of each primer in a 25-µl reaction using the standard MyTaq PCR cycling conditions, but with an initial denaturation time of 2:45 and a 45°C annealing temperature.

Crabs that molted (n = 3), died (n = 2), possessed a double externa (n = 3), were below the target size threshold (n = 1), lost a chela (n = 1), did not consume mussels (n = 60), or whose sex was misidentified in the field (n = 17) were excluded from analyses after post-experiment examination. Non-feeding *E. depressus* were removed from analysis because the effects of captivity potentially cause a loss of appetite and/or reduced foraging (Seed, 1980), and it should be noted that the number of non-feeding *E. depressus* removed was equal between the parasitized and the unparasitized treatments (45% and 46% of the total, respectively). No crabs died during the acclimation period. Statistical analyses were conducted using 43 parasitized and 29 unparasitized *E. depressus*.

Data Analyses

Nonparametric Kruskal-Wallis tests were used to assess biotic (consumption rates among parasitized crabs, consumption rates among unparasitized crabs, CW among parasitized crabs, CW among unparasitized crabs, CW between parasitized and unparasitized crabs) and abiotic (water temperature and salinity) parameters across all experiments to determine if they could be analyzed together. Consumption rates between parasitized and unparasitized *E. depressus* were compared using a Kruskal-Wallis test because the data were not normally distributed and could not be normalized by routine transformations. Data analyses were run in PAST (PAlaeontological STatistics, Hammer et al., 2001) with an *a priori* alpha set at 0.05.

Results

The four experiments were treated as replicates and all data pooled after Kruskal-Wallis tests determined there were no significant differences among biotic and among abiotic parameters across all experiments (Table 3). Parasitized *E. depressus* consumed significantly fewer mussels than unparasitized *E. depressus* (Kruskal Wallis, $H = 5.94$, $d.f. = 1$, $p = 0.02$; Figure 8). The median number of mussels consumed by unparasitized *E. depressus* was 4.0 per 72 hours (4.9 ± 3.7, mean ± SD), while parasitized *E. depressus* consumed a median of only 2.0 mussels (2.7 ± 2.0, mean ± SD).

Discussion

Infection by *L. panopaei* reduced *E. depressus* mussel consumption by a factor of 2. This is the first study to investigate the effects of the invasive rhizocephalan parasite *L. panopaei* on *E. depressus* consumption of bivalves. Previous rhizocephalan–host behavioral studies (e.g. Bishop and Cannon, 1979; Wardle and Tirpak, 1991) in conjunction with this study's consumption data suggest that infected *E. depressus* may be energetically compromised, reducing overall fitness and mobility. Decreased mobility of an infected individual can potentially affect foraging as well as burrowing behavior (Bishop and Cannon, 1979; Wardle and Tirpak, 1991). For example, parasitized *Callinectes sapidus* Rathbun, 1896 are less aggressive when feeding, and rarely burrowed below the sediment (Wardle and Tirpak, 1991). Sand crabs *Portunus pelagicus* (Linnaeus, 1758) infected by the rhizocephalan *Sacculina granifera* Boschma, 1973 buried in sediment at a slightly slower rate than ovigerous female sand crabs and at a significantly slower rate than non-ovigerous sand crabs (Bishop and Cannon, 1979).

Normal feeding behavior is likely interrupted in an infected individual because of mechanical and physiological hindrances. Crevices within oyster shell cultch provide protection for *E. depressus* (Meyer, 1994) and a place for them to forage for small bivalves (McDermott, 1960), but the externae on infected individuals likely complicates maneuverability in the oyster reef interstices. Rhizocephalan–infected *P. pelagicus* exhibit a difference in stance because the externa pushes the exterior portion of the cephalothorax higher (Bishop and Cannon, 1979), which may alter normal feeding behavior. Isaeva et al. (2001) found that the interna of the rhizocephalan *Sacculina polygenea* (Lützen and Takahashi, 1997) surrounds the digestive and reproductive organs

of its brachyuran host *Hemigrapsus sanguineus* (De Haan, 1835). It is possible that internal organ stress, especially those related to feeding, may interfere with a host's ability to forage.

The *Loxothylacus panopaei* invasion may cause trophic changes in intertidal oyster reef ecosystems because the parasite reduces predation intensity in the mud crab-mussel predator-prey relationship. The presence of this invasive rhizocephalan may also change the natural relationship between predators and prey because the parasite arrests host ecdysis and growth for the remainder of their lives. O'Brien and Van Wyk (1985) maintained that rhizocephalan parasites can skew the mud crab population toward smaller individuals that typically consume smaller prey (McDermott, 1960; Seed, 1980). However, *E. depressus* collections by Hines et al. (1997) found infection in crabs 6–18 mm CW, and so support for a smaller-skewed infected population is lacking. Regardless, mussels consumed by crabs within the infected size range may be less likely to be released from predation by *E. depressus* under the infection scenario, creating a bottleneck whereby few mussels survive to reach larger sizes.

Knowledge of *L. panopaei* populations in South Carolina estuaries has, to date, been limited. Hines et al. (1997) did not find parasitized crabs at their South Carolina collection sites (North Inlet and Charleston, 1983, 1986), despite finding *L. panopaei* in Maryland, Virginia, North Carolina, and Florida. Mud crab collections made in North Inlet, South Carolina in the 1970s and 1980s made no mention of infected individuals (McDonald, 1977; Dame and Vernberg, 1982). Collections made for laboratory experiments confirm the presence of *L. panopaei* at Clambank Creek, and as far as we are aware, this is the first report of the parasite in South Carolina waters. This study

demonstrated that *L. panopaei* has a significant negative effect on *E. depressus* prey consumption with clear implications for individual growth and fitness. These data suggest that the *L. panopaei* invasion may alter the ecological role of infected mud crabs and cause changes to the trophic structure of an oyster reef community.

Table 3. Biotic and abiotic data across four feeding experiments were pooled and subsequently tested (Kruskal-Wallis) to examine mussel consumption between parasitized and unparasitized crabs. **A.*** Mussel consumption between parasitized and unparasitized crabs; **B.** Mussel consumption among parasitized crabs; **C.** Mussel consumption among unparasitized crabs; **D.** Carapace width among parasitized crabs; **E.** Carapace width among unparasitized crabs; **F.** Carapace width between parasitized and unparasitized crabs; **G.** Mean daily water temperature among all experiments; **H.** Mean daily salinity among all experiments.

†H is the Kruskal-Wallis test statistic. ††DF is degrees of freedom.

	A*	B	C	D	E	F	G	H
p	0.015	0.668	0.834	0.530	0.712	0.180	0.392	0.392
H†	5.648	1.561	0.866	2.208	1.375	3.877	3.000	3.000
DF††	1	3	3	3	3	1	3	3

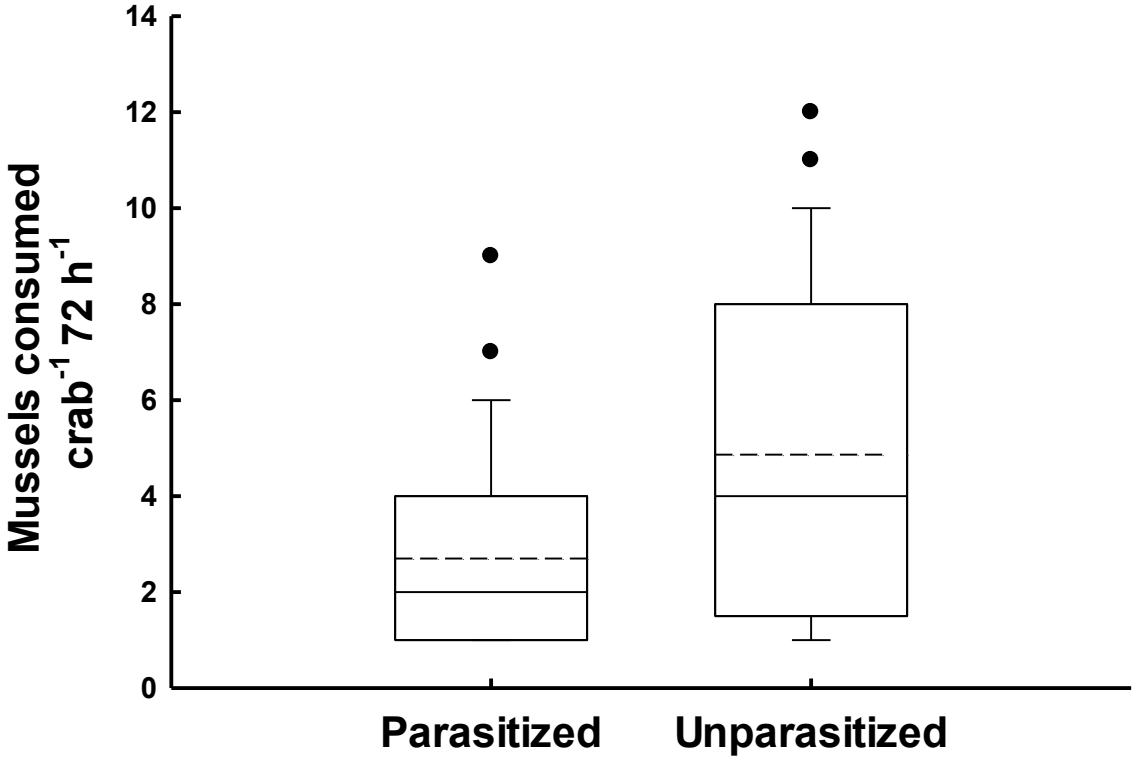


Figure 8. The mean prey consumption was significantly different between parasitized and unparasitized crabs (Kruskal-Wallis, $H = 5.648$, $p = 0.015$). The median number of mussels consumed by parasitized and unparasitized crabs was 2.0 and 4.0, respectively (solid line). The mean number of mussels consumed by parasitized and unparasitized crabs was 2.7 ± 2.0 (mean ± SD) and 4.9 ± 3.7, respectively (dashed line). Black dots represent outliers (parasitized: 7 and 9 mussels; unparasitized: 11 and 12 mussels).

CHAPTER 3:

RELATIONSHIPS AMONG HOST CRAB CARAPACE WIDTH AND SIZE AND COLORATION OF *LOXOTHYLACUS PANOPAEI* EXTERNAL REPRODUCTIVE STRUCTURES

Introduction

Loxothylacus panopaei (Gissler, 1884) is a parasitic rhizocephalan barnacle whose adult form consists of an external reproductive structure (the externa), and an internal rootlet system that absorbs nutrients from its mud crab host (the interna; Walker et al., 1992). A mature externa releases free-swimming, lecithotrophic naupliar larvae into the water column where they metamorphose into non-feeding dioecious cyprid larvae within two days (Glenner, 2001; Walker, 2001). A female cyprid larva settles on a potential crab host within 24 hours of the host molt and penetrates through its gills to the hemocoel. Here the cyprid develops into a parasitic interna (Walker et al., 1992; Glenner, 2001). After 25–42 days, the parasite emerges from the crab at the junction of its thorax and abdomen as a microscopic white sac (Reinhard, 1950; Wardle and Tirpak, 1991) where it awaits fertilization by male cyprid larvae (Glenner, 2001; Walker, 2001).

Loxothylacus panopaei is macroparasitic because reproduction is done outside the host within the parasitic externa (Anderson and May, 1981). A typical *L. panopaei* externa is flattened against the host body with the mantle opening positioned anteriorly. This characteristic shape is common on singly-infected hosts and less common on hosts with multiple infections (Dillon and Zwerner, 1966). Externae have been observed in a variety of colors ranging from white in the youngest to dark brown, purplish or black in

the oldest (Reinhard, 1950; Daugherty, 1969; Wardle and Tirpak, 1991). The changes in externa color are hypothesized to be associated with progressing stages in larval development inside the externa, where the darkest and oldest externa stage contains fully developed naupliar larvae (Reinhard, 1950). The size of the parasitic externae is likely proportional to the host crab size. Wardle and Tirpak (1991) found that the *Loxothylacus texanus* Boschma, 1933 externae on the host *Callinectes sapidus* Rathbun, 1896 were larger as crab carapace width increased. They also found that singly-occurring externae were larger than externae of multiple infections (double, triple or quadruple).

Daugherty (1969) investigated *Loxothylacus panopaei* externa metrics (metrics is used here to describe size and color) in *Eurypanopeus depressus* (Smith) and *Rhithropanopeus harrisii* (Gould, 1841) hosts in Chesapeake Bay early in the invasion, but no follow-up study has been conducted. Wardle and Tirpak (1991) made similar size and color observations but of *Loxothylacus texanus* on *Callinectes sapidus*. These observations might be difficult to extend to the *L. panopaei–E. depressus* relationship because infected *C. sapidus* are larger (49–100 mm CW; Wardle and Tirpak, 1991) than infected *E. depressus* (5.8–16.3 mm CW). This study was conducted to contribute a current report on externa characteristics of North and South Carolina *Loxothylacus panopaei*. The objective of this study was to investigate relationships between the following: (1) size and color of *L. panopaei* externa, (2) size of *L. panopaei* externa and *E. depressus* carapace width, and (3) color of *L. panopaei* externa and *E. depressus* carapace width.

Materials and Methods

North Carolina Collection

Eurypanopeus depressus mud crabs infected with *Loxothylacus panopaei* were collected by hand from intertidal oyster reefs (Hines et al., 1997) at three sites in North Carolina. Two of these sites were located within the Masonboro Island and Rachel Carson National Estuarine Research Reserves, respectively. The Masonboro Island site is a fringing oyster reef in Loosins Creek, New Hanover County (34°10′21″N, 77°49′57″W), adjacent to the Atlantic Intracoastal Waterway. Infected crabs were collected from a fringing oyster reef in the Rachel Carson Reserve along the Taylor's Creek Channel (34°42′46″N, 76°40′23″W) adjacent to the Duke University Marine Laboratory in Carteret County. The third collection site was a small jetty in Bogue Sound ((34°43′35″N, 76°49′15″W), in Morehead City, Carteret County.

South Carolina Collection

Monthly collections (January 2012–January 2013) of xanthid crabs were made by hand (Hines et al., 1997) from intertidal oyster reefs in Dunn Sound at Waties Island (33°51′11″N, 78°35′37″W), Murrells Inlet at Garden City Causeway (33°34′45″N, 79°00′14″W), and Clambank Creek in the North Inlet-Winyah Bay National Estuarine Research Reserve (33°20′04″N, 79°11′33″W). Sites included a patch reef of 875 m² in Dunn Sound, a patch reef of 240 m² at Garden City Causeway, and a thin stretch of fringing reef approximately 575 m long in Clambank Creek.

A single collection consisted of excavating all surface oyster clusters, buried shell and aerobic sediment from a 0.25 m^2 area. The excavated material was placed into a bin and clusters were broken apart by hand to ensure xanthid crabs of all sizes were captured. This process was repeated until approximately 100 xanthid crabs were collected. Between December–March, mud crabs are less dense in the intertidal oyster reef (Dame and Vernburg, 1982), and so the target collection was reduced to 50 crabs. Each collection started from an adjacent undisturbed area on the reef, and subsequent 0.25 m^2 areas during a collection were sampled every 3 m along the lower intertidal zone parallel to shore.

All crabs collected from North and South Carolina were placed into plastic containers and transported to the laboratory where they were frozen at or below 0°C until measurement and examination. In the laboratory, all crabs were sexed and identified to species using external morphology (Williams, 1984). The abdominal flap of each crab was separated from the body and examined for the presence of an externa using a dissecting microscope. Crabs were classified as parasitized if an externa of any size was present (virgin or mature). Virgin externae are unfertilized translucent external sacs, while mature externae are fertilized external sacs that range in color from tan to purple and are visible to the naked eye (Figure 9; Walker et al., 1992). Maximum crab carapace width (CW) was measured to the nearest 0.1 mm using a digital caliper.

While variability exists in the quantification of externa color in the Rhizocephala, Wardle and Tirpak (1991) showed that using a pocket color wheel (The Color Wheel Company, Philomath, OR, USA) for comparison to externa color has yielded consistent results when viewed under a dissecting microscope, and so their method was adopted in

this coloration study. All externae were placed into one of six color groups in accordance with *Loxothylacus texanus* externa color groupings designated by Wardle and Tirpak (1991): (1) white (youngest), (2) tan, (3) yellow, (4) brown, (5) salmon, and (6) purple (oldest).

Externa-bearing *E. depressus* were placed on a ruler abdomen up and photographed using a high resolution (1260 x 1260 dpi) Nikon digital SLR camera. Ten singly infected *E. depressus* with mature externae from the April–July collections at five of the six locations in North and South Carolina were randomly selected for analyses. The Masonboro Island collection was the exception where nine *E. depressus* were analyzed, because there were only nine infected individuals found. At least one *E. depressus* selected for analysis exhibited a double externa at each location, but the Bogue Sound Jetty collection had three crabs with double infections, and the Waties Island collection had two crabs with double infections included in analysis. Because the magnification was specimen specific, the mm lines on each picture's ruler were used to calculate the actual dimensions (mm). The left-right axis (horizontal plane, perpendicular to anterior-posterior axis; Wardle and Tirpak, 1991) of each externa was measured to the nearest 0.1 mm.

Analyses

Externae size and color were compared using a nonparametric Kruskal-Wallis test because the data did not satisfy the ANOVA assumptions of homogeneity of variance and normality even with transformations. A Spearman rank order correlation was performed to compare host crab CW and *L. panopaei* externa size because CW data were not

normally distributed (Shapiro-Wilk). A Kruskal-Wallis test was run to compare externae color and *E. depressus* CW because data were not normally distributed (Shapiro-Wilk). All statistical analyses were run in SigmaPlot v.12.0 (Systat Software, Inc., San Jose, CA) with an *a priori* set at 0.05.

Results

In total, 476 *L. panopaei* external reproductive structures were analyzed from North and South Carolina *E. depressus*. Four hundred and forty-five infected *E. depressus* were collected between January 2012–January 2013: 391 from South Carolina and 54 from North Carolina. Of the 391 infected *E. depressus* collected from South Carolina, 385 were analyzed for host size and externae color, while all 54 of the North Carolina infected *E. depressus* were analyzed for host size and externae color. In the South Carolina collection, 26 *E. depressus* had double externae (6.8%) and one *E. depressus* had triple externae (0.3%) for a total of 413 *L. panopaei* analyzed. Four infected *E. depressus* in the North Carolina collection had double externae (7.4%), one had a triple externae (1.9%) and one had four externae (1.9%), for a total of 63 *L. panopaei* analyzed.

The most frequent externa color in the North Carolina collection was yellow (33.3%; Table 4; Figure 10), while purple (oldest) was the most abundant in South Carolina collections (37.8%), as well as in the North and South Carolina collections combined (35.5%). Purple externa never represented less than 25% of the month's collection, except in September 2012 when it composed 23.1% of the collection. In November and December 2012, purple externae composed 50% of the monthly collections.

The mean left-right axis size for each externa color group were as follows: tan, 6.1 mm ± 1.3 (mean ± SD); yellow, 4.7 mm ± 1.5; brown, 7.1 mm ± 1.6; salmon, 5.6 mm ± 2.0; and purple, 6.7 mm ± 1.0. The yellow externa color group was significantly smaller than the tan (Mann-Whitney-U; $U(1) = 78$, $z = -2.97$, $p = 0.003$), brown ($U(1) = 15$, $z = -2.88$, $p = 0.004$) and salmon ($U(1) = 52$, $z = -3.73$, $p < 0.001$) color groups. White externae were too small to measure with current methods.

The data pooled across months and sites showed that the left-right axis of *L. panopaei* externa increased with increasing host CW in *E. depressus* exhibiting one externa from April–July 2012 (Spearman rank order correlation; $p < 0.001$, $r = 0.65$; Figure 11). Externae size ranged from 3.9–9.8 mm, and infected *E. depressus* CW ranged from 7.2–16.3 mm, although the crab with CW 16.3 mm exhibited an externa of only 5.0 mm. The smallest *E. depressus* infected (7.2 mm CW) exhibited the smallest externa (3.9 mm).

The left-right axes of externae in *E. depressus* with double infections were significantly smaller than externae in *E. depressus* with single infections (Kruskal-Wallis; $H = 32.4$, $d.f. = 1$, $p < 0.001$). Double externa represented 30 of 445 total infections in North and South Carolina. The mean size of externae in hosts with double infections was 4.0 mm ± 1.1 (mean ± SD), while the mean size of externae in hosts with single infections was 6.6 mm ± 1.2.

There was no relationship between externae color and *E. depressus* CW in the total collection (North and South Carolina). The mean CW ± SD for each color group were as follows: white, 9.7 mm ± 1.4; tan, 9.8 mm ± 1.9; yellow, 10.1 mm ± 1.9; brown, 9.7 mm ± 1.5; salmon, 9.9 mm ± 1.7; purple, 9.9 mm ± 1.3. In the South Carolina collection, there were no significant differences in *E. depressus* CW among *L. panopaei* externa

color (Kruskal-Wallis, $p > 0.05$), but in the North Carolina collection, the mean CW of crabs with yellow externae (9.4 mm ± 1.4) were significantly smaller than the mean CW of crabs with salmon externae (11.0 mm ± 1.4; Mann-Whitney-U; U(1) = 52, z = -2.75, $p = 0.006$) and purple externae (10.9 mm ± 1.4; Mann-Whitney-U; U(1) = 44.5, z = -3.25, $p = 0.012$).

Discussion

The purple color group was the most frequent color in the total collection (North and South Carolina). Lützen (1984) found that most *Sacculina carcini* Thompson, 1836 externae died and fell off their hosts before reaching one year old. Rhizocephala in the family Petrogastridae that survive past one year grow at variable rates and typically die between 3–5 years old (Lützen, 1987). There is little known about age determination in *L. panopaei*, but it is known that the purple color group is the final stage in externa development (Reinhard, 1950; Daugherty, 1969; Wardle and Tirpak, 1991), and therefore this group might accumulate more members than color groups that are transitional stages, (white through salmon).

Daugherty (1969) found that darker purple externae were more common in the winter, and lighter externae were found more often in the summer. *Loxothylacus panopaei* larval nauplii are released from the reproductive external sac into the water column, and within two days metamorphose into cyprid larvae around 25°C (typically late May or early June in North Inlet, SC; National Oceanic and Atmospheric Administration, Office of Ocean and Coastal Resource Management, NERRS System-wide Monitoring Program, 2004; Glenner, 2001; Walker, 2001). Female cyprids settle on *E. depressus* hosts, inoculating individuals within 48–72 hours, and emerging from the crab abdomen as a virgin externa

within 25–42 days (Walker et al., 1992; Alvarez et al., 1995; Glenner, 2001). With exposure to male cyprid larvae, fertilization can start almost immediately after externa emergence, and lasts approximately 15 days (Alvarez et al., 1995). Following these observations, if a cyprid larva settles on a host at the beginning of June, the virgin externa should emerge from host abdomen in July, become fertilized by the end of July, leading to an abundance of white virgin or immature externae during late August–September in Carolina tidal creeks. In contrast, the highest frequency of white externae were observed in March (46.2%), while low frequencies (5.0–13.0%) were observed from April–August. The breeding biology of *L. panopaei* is largely unknown from field studies, as reproduction and growth rate knowledge is limited to laboratory studies, and the parasite might behave differently outside of a natural setting.

The tan color group had a significantly larger left-right axis than the yellow color group, which differed from results in Wardle and Tirpak (1991) who found that externae size increased as externae transitioned through the color stages from white to tan to yellow. Mean externa size from this study decreased from tan to yellow, increased from yellow to brown, decreased from brown to salmon and increased from salmon to the final purple stage. Daugherty (1969) measured 19 live *L. panopaei* externae on *E. depressus* periodically for four months, and observed that changes in externae size were rarely consistently increasing from white to reddish-brown to light brown. He attributed most changes in externae size to temporary contraction of the sac, reproductive stage, or measurement error, rather than true growth.

The left-right axes of singly-occurring externae tended to increase with increasing host CW. These results support a similar trend observed by Wardle and Tirpak (1991), where

singly-occurring *L. texanus* externae increased in size with increasing *Callinectes sapidus* CW. They hypothesized that the parasitic externa grows based on two principles that need to be in balance for its survival: (1) The externa must be large enough to produce nauplii in amounts that would allow successful infection of new hosts; and (2) the externa must be small enough to not interfere with the host's ability to gain nutrition, as well as remain attached to the host's abdomen. The second point here might also include avoiding host predation, as a large externa might disrupt the host's ability to escape predators.

Externae involved in double infections were smaller than those of single infections most likely because the increase in number of externae at the host abdomen decreases available growth space. These results are in concordance with results from Wardle and Tirpak (1991). They hypothesized that these multiple externae were actually multiple parasites, and therefore competition for space and nutrition must be considered. A parasite puts a nutritional demand on its host, and so if multiple infections occur, each parasite must remain small enough so as not to demand too much from its host.

The apparent difference in CW between *E. depressus* with yellow externae and *E. depressus* with salmon and purple externae in the North Carolina collection is surprising because the externa stage (indicated by color) is not dependent on host size. These differences can likely be attributed to a small North Carolina sample size (n = 63), and so the total collection (North and South Carolina) should be used as representative of the true *L. panopaei* population. There were no significant differences in CW between color groups in the total collection, supporting earlier studies that concluded *L. panopaei* causes parasitic anecdysis—the cessation of molting—with a rhizocephalan infection

(O'Brien and VanWyk, 1985; O'Brien and Skinner, 1990). Thus CW does not increase as externae develop through the color stages and sizes (Reinhard, 1950; Wardle and Tirpak, 1991).

Table 4. Externa color frequency distribution of *Loxothylacus panopaei* infecting *Eurypanopeus depressus* in North and South Carolina. The total number of specimens per state is indicated in the last row, while the total column shows the total number and frequency of each externa color. The numbers in parentheses indicate the sample size.

Color of externa	**NC**	**SC**	**Total**
White	3.2 (2)	17.9 (74)	16.0 (76)
Tan	17.5 (11)	15.3 (63)	15.5 (74)
Yellow	33.3 (21)	16 (66)	18.3 (87)
Brown	6.3 (4)	6.5 (27)	6.5 (31)
Salmon	19.0 (12)	6.5 (27)	8.2 (39)
Purple	20.6 (13)	37.8 (156)	35.5 (169)
	n = 63	**n = 413**	**n = 476**

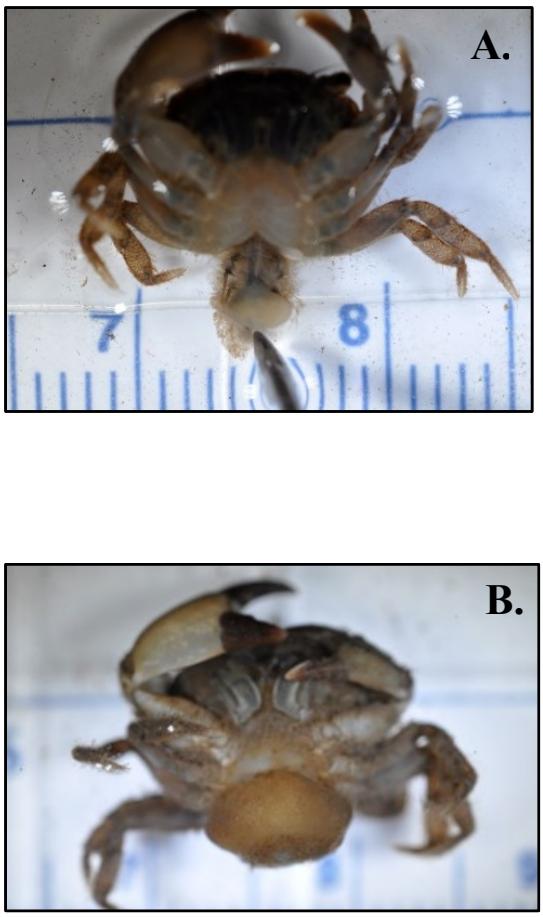


Figure 9. Eurypanopeus depressus hosts were classified as parasitized by Loxothylacus panopaei if an externa of any size was present on the abdomen. **A.** Translucent unfertilized virgin externa. **B**. Fertilized mature externa.

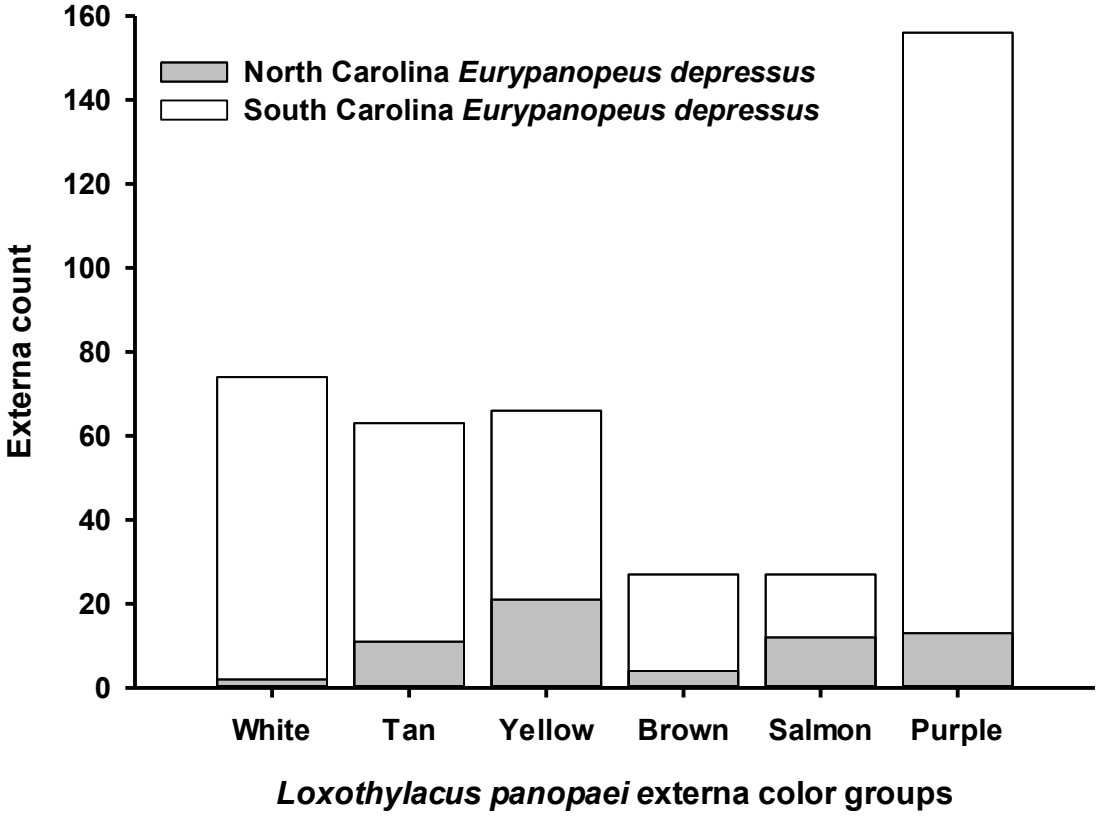


Figure 10. Number of *Loxothylacus panopaei* externae in each color group. Total number of externae analyzed was 476. Development of externae progresses from white in the youngest (n = 76), to tan (n = 74), yellow (n = 87), brown (n = 31), salmon (n = 39) and purple in the oldest (n = 169). Purple externae were the most frequent (35.5%) in the total collection.

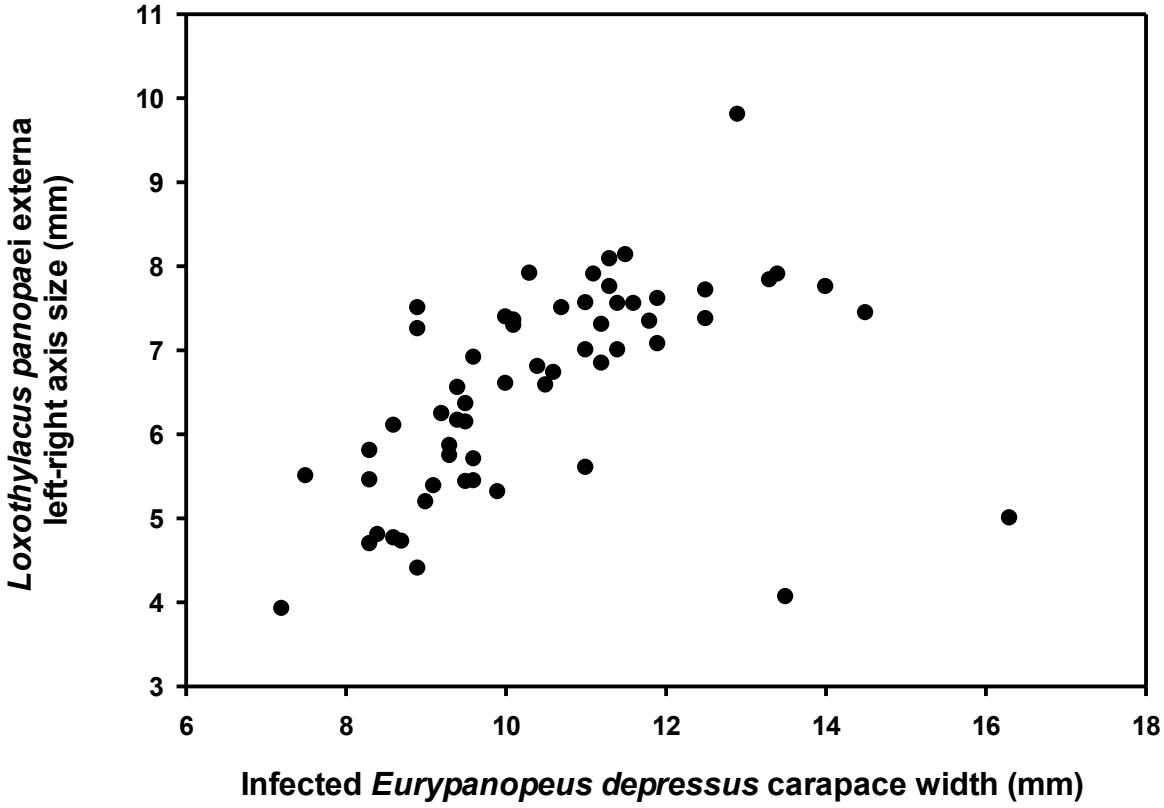


Figure 11. Size of *Loxothylacus panopaei* externa left-right axis (mm) increased with increasing *Eurypanopeus depressus* carapace width (Spearman rank order correlation; $p < 0.001$, $r = 0.65$) in North and South Carolina parasite populations.

SUMMARY

This thesis provides the first report of *Loxothylacus panopaei* (Gissler, 1884) from Waties Island, Murrells Inlet, and North Inlet, South Carolina, and Masonboro Island, North Carolina. The observed parasite prevalences are consistent with prevalences from the invasive range recorded from Long Island Sound, Chesapeake Bay, coastal Georgia and the Atlantic coast of Florida north of Cape Canaveral (Daugherty, 1969; Kruse and Hare, 2007; Kruse et al., 2012; Freeman et al., 2013). Generally, invasive range prevalences were higher than prevalences recorded from the native range, and might be indicative of epidemic infection in recently (approximately 20 years) invaded areas (Hines et al., 1997; Snyder and Evans, 2006; Kruse and Hare, 2007; Hulme, 2009).

This study was the first to generate North and South Carolina *L. panopaei* COI sequence data, which fills the geographic gap in current DNA sequence data generated by Kruse and Hare (2007) and Kruse et al. (2012) within the parasite's invasive range. *Loxothylacus panopaei* H_1 and H_2 were found only in the invasive range (Chesapeake Bay–northern Florida), while H_3 consisted of *L. panopaei* from both the invasive and native ranges (Kruse et al., 2012). The previously unidentified H_{NC}, found in the Rachel Carson Reserve, North Carolina, is closest to a Chauvin, Louisiana sequence reported by Kruse et al. (2012).

These data support two hypotheses about the source of North and South Carolina *L. panopaei* populations: (1) Some of the North and South Carolina parasites in H_3 and the single H_{NC} are a direct result of human-mediated translocation of mud crabs from the Gulf of Mexico to the Carolinas; or (2) these parasites represent a southern range expansion from the Chesapeake Bay invasive population. It is most likely that our

analyses indicate that North and South Carolina *L. panopaei* populations are a result of the Chesapeake Bay parasite population range expansion south, which continues through to northern Florida. Because all six of our collection locations were not closer than 30 km from each other (besides the Rachel Carson Reserve and the Bogue Sound Jetty sites that were located in Bogue Sound, approximately 9 km apart), spread of *L. panopaei* is possibly dependent on ballast water exchange and biofouling on vessels that move between estuaries (Cohen and Carlton, 1997; Davidson et al., 2008; Kerckhof et al., 2010). Davidson et al. (2008) observed mud crabs infected with *L. panopaei* associated with fouling bryozoa and bivalves on the vessel, ORION, and so this form of transport might be a vector for range expansion in the invasive range.

This is the first investigation into the effects of *L. panopaei* on *E. depressus* consumption of bivalves. Infection reduced *E. depressus* mussel consumption by a factor of two. Results from this experiment in conjunction with previous rhizocephalan–host behavioral studies suggest that infected *E. depressus* may be energetically compromised, reducing overall fitness and mobility. In a laboratory study, *Callinectes sapidus* Rathbun, 1896 infected with *Loxothylacus texanus* Boschma, 1933 foraged less aggressively than uninfected crabs, and infected *C. sapidus* burrowed at a slower rate than uninfected crabs (Wardle and Tirpak, 1991). Reduction in host fitness might create a situation where infected *E. depressus* are unable to escape predation, resulting in a decline in host populations in oyster reef habitats.

High prevalence of *L. panopaei* effects *E. depressus* in two ways: (1) Host abundance on oyster reefs may be reduced due to parasitic castration (Van Engle et al., 1966); and (2) infected *E. depressus* consume fewer mussels than uninfected crabs. Both situations

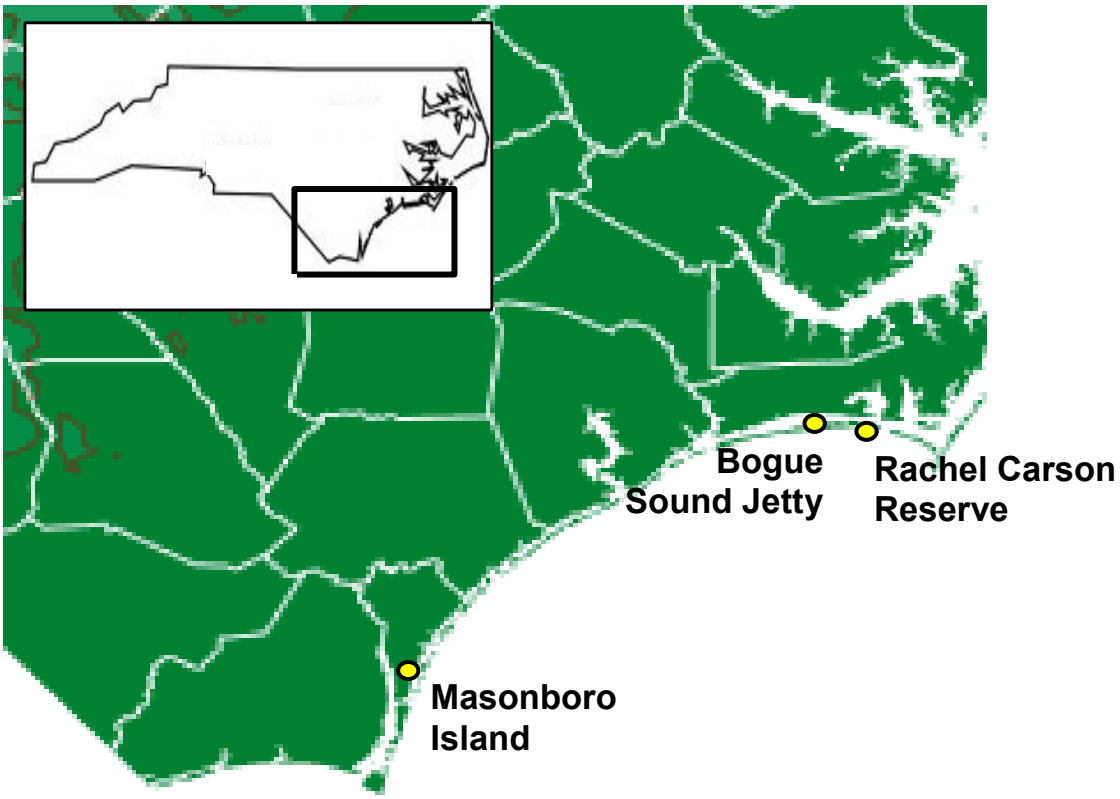
potentially affect reef-associated bivalve populations. Mud crabs are an important source of juvenile mussel and oyster spat mortality (Seed, 1980; Kulp et al., 2011), and so the presence of the parasite alters the predator-prey relationship. *Loxothylacus panopaei* infects *E. depressus* 5.8–16.3 mm CW, and so bivalves targeted as prey by this host size range might be positively affected where *L. panopaei* is highly prevalent. Monitoring bivalve populations on oyster reefs where *L. panopaei* is present might provide insight into the parasite's broader ecological effects in the invasive range. More conclusive statements can only be made after investigations of reef-associated bivalves have been conducted.

This thesis provides a current description of the relationships among host crab carapace width, and size and color of *L. panopaei* external reproductive structures on *E. depressus*. We found the purple color group to be the most frequent color in the total collection (North and South Carolina) during the 13 month study. This study's results were consistent with trends observed by Wardle and Tirpak (1991), where singly-occurring externae increased in size with increasing crab CW. Because larger externae likely support larger larval broods (Wardle and Tirpak, 1991), it would benefit the parasite to infect larger hosts.

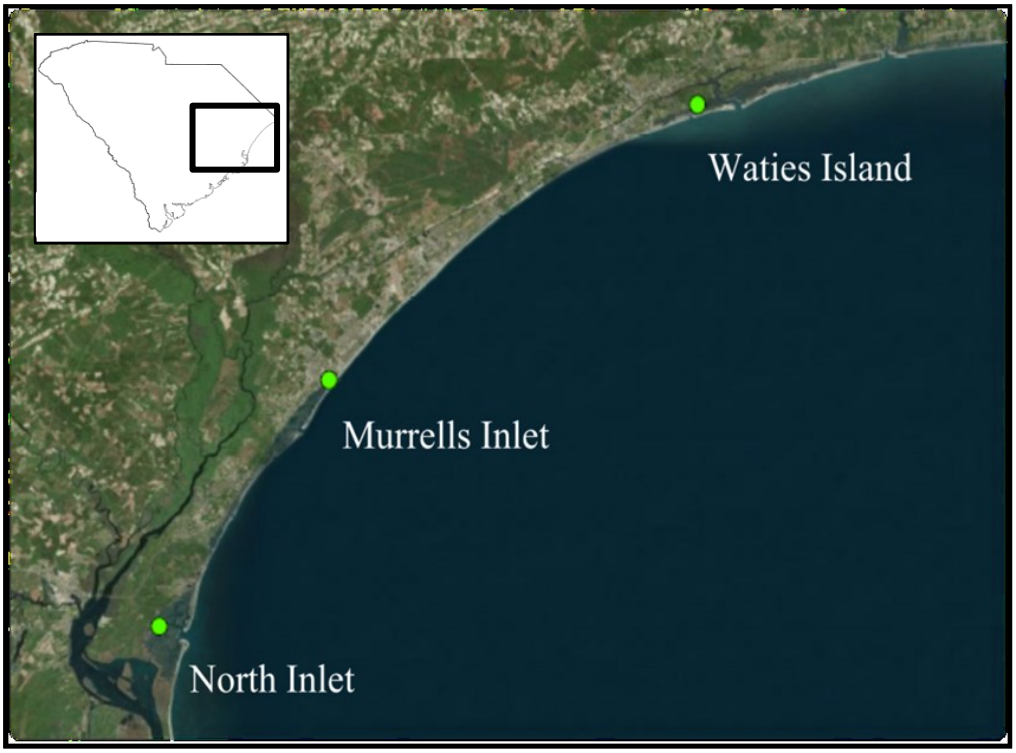
A critical geographic gap in *Loxothylacus panopaei* prevalence, genetic and behavioral data has been filled. South Carolina prevalence data are consistent with previous prevalences reported from the invasive range. *Loxothylacus panopaei* COI sequence data generated here is available in GenBank, and can be used to support a range expansion south from the Chesapeake Bay invasive population. This study showed that

foraging is compromised in infected *Eurypanopeus depressus*, suggesting *L. panopaei* has broader ecological effects that are yet unmeasured in the parasite's invasive range.

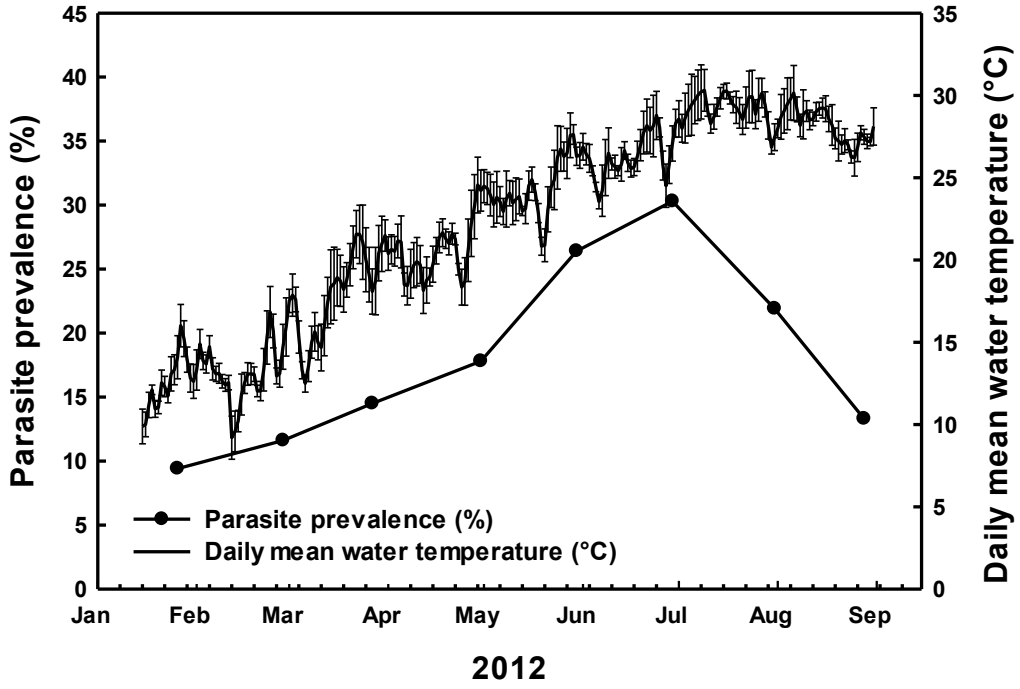
APPENDIX



Appendix, Figure 1. North Carolina collection locations from north to south: Rachel Carson Reserve in Beaufort, Bogue Sound Jetty in Morehead City, and Masonboro Island in New Hanover County.



Appendix, Figure 2. South Carolina collection locations from north to south: Dunn Sound at Waties Island, Murrells Inlet at Garden City Causeway, and Clambank Creek in North Inlet.



Appendix, Figure 3. Prevalence of *Loxothylacus panopaei* in host *Eurypanopeus depressus* at Clambank Creek, North Inlet, South Carolina January–August 2012 and associated daily mean water temperatures (ºC). Error bars represent standard deviation. Prevalence increased with increasing water temperatures from January–July 2012, then decreased as water temperatures remained stable.

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